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**AMENDED APPEAL BRIEF**

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## AMENDED APPEAL BRIEF

Sir:

Appellants hereby submit an original and two copies of this Amended Appeal Brief to the Board of Patent Appeals and Interferences ("the Board") in response to the Communication which was mailed on March 23, 2006 ("the Communication"), which indicates that the Appeal Brief filed on December 28, 2005, is not acceptable because:

Appellant indicates in the Brief that there are no related appeals or interferences. However, the Examiner is aware that there are appeals which may be related to, directly affect or be directly be affected by or have a bearing on the Board's decision in the pending appeal. The Examiner does not know the application numbers. Appellant is required to submit a statement identifying by application, patent, appeal or interference number all other prior and pending appeals, interferences or judicial proceedings known to appellant, the appellant's legal representative, or assignee which may be related to, directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

A telephone interview ("the interview") was conducted on May 22, 2006, between Appellants' representative David Hibler and Examiner Sandra Wegert to discuss the Communication. During the interview, Appellants' representative David Hibler reiterated to Examiner Wegert that while Appellants have a number of prior and pending appeals before the Board, none of these appeals concerned the subject matter of the present appeal. Therefore, Appellants' statement in the Brief that "there are no related appeals or interferences" is in fact correct. However, to avoid any further delays, Examiner Wegert reviewed Appellants' Brief to ensure compliance with all applicable rules, and concluded that the text of Section VI should be in the form of a statement rather than a question, and that this would also apply to subheading A in Section VII. Therefore, the present Amended Appeal Brief is submitted to overcome these objections.

This Amended Appeal Brief is due on April 23, 2006, and is timely submitted in light of the concurrently filed Petition for an Extension of Time of one month to and including May 23, 2006, and authorization to deduct the fee as required under 37 C.F.R. § 1.17(a)(1) from Appellants' Representatives' deposit account. Appellants believe that no fees in addition to the fee for the extension of time are due in connection with this Amended Appeal Brief. However, should any additional fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason related to this

communication, the Commissioner is hereby authorized to charge any underpayment or credit any overpayment to Lexicon Genetics Incorporated Deposit Account No. 50-0892.

**I. REAL PARTY IN INTEREST**

The real party in interest is the Assignee, Lexicon Genetics Incorporated, 8800 Technology Forest Place, The Woodlands, Texas, 77381.

**II. RELATED APPEALS AND INTERFERENCES**

Appellants know of no related appeals or interferences.

**III. STATUS OF CLAIMS**

The present application was filed on August 28, 2001, claiming the benefit of U.S. Provisional Application Number 60/230,178, which was filed on September 1, 2000, and included original claims 1-3. A Restriction and Election Requirement was issued on November 20, 2003, separating the original claims into two separate and distinct inventions. In a response to the Restriction and Election Requirement submitted to the Office on December 19, 2003, Appellants elected without traverse the claims of the Group I invention (original claims 1 and 2) for prosecution on the merits, cancelled claim 3 without prejudice and without disclaimer as drawn to a non-elected invention, and added new claims 4-7.

A First Official Action on the merits ("the First Action") was issued on April 28, 2004, in which claims 1, 2, and 4-7 were rejected under 35 U.S.C. § 101 as allegedly lacking a patentable utility, claims 1, 2, and 4-7 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly unusable by the skilled artisan due to the alleged lack of patentable utility, and claim 1 was rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite. In a response to the First Official Action submitted to the Office on July 28, 2004 ("the Response to the First Action"), Appellants cancelled claim 1 without prejudice and without disclaimer, and addressed the rejections of claims 1, 2, and 4-7.

A Second Official Action on the merits ("the Second Action") was mailed on November 17, 2004, indicating that the rejection of claims 1, 2, and 4-7 under 35 U.S.C. § 101 as allegedly lacking a patentable utility, claims 1, 2, and 4-7 under 35 U.S.C. § 112, first paragraph, as

allegedly unusable by the skilled artisan due to the alleged lack of patentable utility, and claim 1 under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite, had been overcome by the amendments and remarks submitted in the Response to the First Action, thus rendering claims 2 and 4-6 allowed, but setting forth a new rejection of claim 7 under 35 U.S.C. § 112, first paragraph, as allegedly not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. In a response to the Second Action submitted on February 15, 2005 (“the Response to the Second Action”), Appellants addressed the rejection of claim 7.

The Final Action was mailed on June 21, 2005, maintaining the rejection of claim 7 under 35 U.S.C. § 112, first paragraph, as allegedly not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. In a response to the Final Action submitted on September 21, 2005 (“the Response to the Final Action”), Appellants once again addressed the rejection of claim 7.

To date, neither an Advisory Action nor a Notice of Allowance has been received by Appellants, who therefore assume that the rejection of claim 7 under 35 U.S.C. § 112, first paragraph, as allegedly not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention, as set forth in the Final Action, has been maintained in light of the Response to the Final Action. Therefore, claim 7 is the sole subject of this appeal. A copy of the appealed claim is included below in the Appendix (Section IX).

#### **IV. STATUS OF AMENDMENTS**

As no amendments subsequent to the Final Action have been filed, Appellants believe that no outstanding amendments exist.

#### **V. SUMMARY OF CLAIMED SUBJECT MATTER**

The present invention relates to Appellants’ discovery and identification of novel human polynucleotide sequences that encode a protein sharing sequence similarity with mammalian transporter proteins (see, at least, the specification at page 1, lines 9-12), and more particularly shares structural similarity with mammalian GABA transporters (see, at least, the specification at page 2, lines 2-4). The

present claims are directed to polynucleotide sequences that encode the disclosed GABA transporter protein as set forth in SEQ ID NO:2, including the polynucleotide sequence of SEQ ID NO:1, expression vectors comprising these polynucleotide sequences, and host cells comprising these expression vectors. The presently claimed polynucleotide sequence was obtained from clustered ESTs, genomic sequence, and cDNA clones from human brain and pituitary gland cDNA libraries (specification at page 16, lines 19-21).

## **VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL**

Claim 7 stands rejected under 35 U.S.C. § 112, first paragraph, as allegedly not enabled.

## **VII. ARGUMENT**

The Final Action rejects claim 7 under 35 U.S.C. § 112, first paragraph, as allegedly not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

In the Second Action, the Examiner stated that claim 7 has been “interpreted ... as reading on isolated host cells, as well as host cells in the context of a multicellular, transgenic organism and host cells intended for gene therapy” (the Second Action at page 3), and, although alluded to in the Second Action (note multiple references to “non-human transgenic animals” on page 4 of the Second Action), specifically noted in the Final Action that “(t)he Specification specifically excludes humans in its discussions of transgenic animals” (the Final Action at page 4). Appellants agree with the Examiner that claim 7 reads on both isolated host cells and host cells within multicellular organisms, which includes non-human transgenic animals and animals receiving gene therapy. The Examiner asserts that claim 7 is not enabled with regard to non-human transgenic animals and gene therapy. Appellants address each of these assertions in turn.

With regard to the enablement of non-human transgenic animals, in the Second Action the Examiner stated that “(t)he specification of the instant application teaches that SEQ ID NO:1 can be expressed in transgenic animals and any technique known in the art may be used to introduce an NHP transgene into animals to produce the founder lines of transgenic animals”, but that claim 7 is not enabled for non-human transgenic animals because “there are no methods or working examples disclosed in the instant application whereby a multicellular animal with the incorporated (‘knocked-in’)



NHP gene of SEQ ID NO:1 is demonstrated to express the NHP peptide”, and “(t)he unpredictability of the art is *very high* with regards to making transgenic animals” (the Second Action at page 3, emphasis in original). With regard to the Examiner’s first argument, Appellants respectfully pointed out in the Response to the Second Action that this argument is not dispositive as to the question of enablement, for it has long been established that “there is no statutory requirement for the disclosure of a specific example” (*In re Gay*, 309 F.2d 769, 135 USPQ 311 (CCPA, 1962)). Thus, this argument alone cannot support an allegation that claim 7 is not enabled.

With regard to the Examiner’s second argument, concerning the unpredictability in the art with regard to making transgenic animals, the Examiner cited four scientific articles that allegedly support this position. Specifically, the Examiner cited Wang *et al.* (*Nuc. Acids Res.* **27**:4609-4618, 1999) and Kaufman *et al.* (*Blood* **94**:3178-3184, 1999) to support the argument that expression levels of an inserted transgene are highly variable, Wigley *et al.* (*Reprod. Fert. Dev.* **6**:585-588, 1994) to support the argument that production of non-human transgenic animals by pronuclear microinjection (one of the methods of producing non-human transgenic animals specifically cited in the specification as originally filed) suffers from limitations such as low frequency of integration events and random integration, and Campbell *et al.* (*Theriology* **47**:63-72, 1997) to support the argument that production of non-human transgenic animals by from ES cells (another method of producing non-human transgenic animals specifically cited in the specification as originally filed) has been difficult. Thus, the Examiner concluded that “it would have required undue experimentation for the skilled artisan to have made any and all transgenic non-human animals according to the instant invention” (the Second Action at page 4).

Rather than list the numerous deficiencies of each of the articles cited by the Examiner, Appellants instead presented evidence in the Response to the Second Action of the state of the art with regard to making transgenic animals as of the filing date of the present application (August 28, 2001), or the filing date of the priority application (September 1, 2000). The specification as originally filed, at page 17, lines 7-10, details that “(a)nimals of any species, including, but not limited to, worms, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, birds, goats, and non-human primates, *e.g.*, baboons, monkeys, and chimpanzees may be used to generate NHP transgenic animals”. Appellants respectfully pointed out that there are **numerous** examples of transgenic worms (nematodes), mice, rats, rabbits, guinea pigs, pigs, birds (chickens), goats and monkeys, years and sometimes decades prior to the filing date of the present application and the priority application. However, rather than provide hundreds of

citations of transgenic animals that were described in the art prior to the filing date of the present or priority applications, Appellants instead respectfully pointed out that, to the best of their knowledge, the first report of a transgenic nematode was in 1988 (Spieth *et al.*, *Dev. Biol.* **130**:285-293; copy of abstract provided in **Exhibit A**), the first report of a transgenic mouse was in 1980 (Gordon *et al.*, *Proc. Natl. Acad. Sci. USA* **77**:7380-7384; copy of manuscript provided in **Exhibit B**), the first report of a transgenic rat was in 1990 (Mullins *et al.*, *Nature* **344**:541-544; copy of abstract provided in **Exhibit C**), the first report of a transgenic rabbit was in 1985 (Hammer *et al.*, *Nature* **315**:680-683; copy of abstract provided in **Exhibit D**), a report of the production of human interleukin-2 in the milk of transgenic rabbits was published in 1990 (Bühler *et al.*, *Bio/Technology* **8**:140-143; copy of abstract provided in **Exhibit E**), the first reports of transgenic guinea pigs were in June and December of 2000 (Suzuki *et al.*, *Gene Ther.* **7**:1046-1054, and Yagi *et al.*, *JARO* **1**:315-325; copies of abstracts provided in **Exhibit F**), a report of the production of human growth hormone in the milk of transgenic guinea pigs was published in 2000 (Hens *et al.*, *Biochim. Biophys. Acta* **1523**:161-171; copy of abstract provided in **Exhibit G**), the first report of a transgenic pig was in 1985 (*see Exhibit D*), a report of the production of a heterologous milk protein in the milk of transgenic pigs was published in 1991 (Wall *et al.*, *Proc. Natl. Acad. Sci. USA* **88**:1696-1700; copy of manuscript provided in **Exhibit H**), the first reports of transgenic chickens were in 1987 (Salter *et al.*, *Virology* **157**:236-240; copy of abstract provided in **Exhibit I**) and 1989 (Bosselman *et al.*, *J. Virol.* **63**:2680-2689; copy of abstract provided in **Exhibit J**), the first reports of transgenic goats were in 1991 (Ebert *et al.*, *Bio/Technology* **9**:835-838, and Denman *et al.*, *Bio/Technology* **9**:839-843; copies of abstracts provided in **Exhibit K**), and the first report of a transgenic monkey (rhesus monkey) was in January of 2001 (Chan *et al.*, *Science* **291**:309-312; copy of manuscript provided in **Exhibit L**). Additionally, the first report of a transgenic cow (raised by the Examiner on page 4 of the Second Action) was in 1991 (Krimpenfort *et al.*, *Bio/Technology* **9**:844-847; copy of abstract provided in **Exhibit M**), the first report of a transgenic sheep (another example of a transgenic mammal) was in 1988 (Simons *et al.*, *Bio/Technology* **6**:179-183; copy of abstract provided in **Exhibit N**), and a report of the production of human anti-hemophilic factor IX in the milk of transgenic sheep was published in 1989 (Clark *et al.*, *Bio/Technology* **7**:487-492; copy of abstract provided in **Exhibit O**). Given the hundreds of reports of non-human transgenic animals prior to the filing of the present or priority applications, of which the reports listed above are only the first examples, Appellants pointed

out that there can be no doubt that the making of non-human transgenic animals was clearly enabled to those of skill in the art at the time the present and priority applications were filed, which is all that is required to meet the enablement requirement under 35 U.S.C. § 112, first paragraph.

The Examiner appears to believe that claim 7 is not enabled for transgenic animals because certain aspects of transgenic technology (expression levels, site-specific *versus* random integration) require undue experimentation to perfect. However, Appellants respectfully pointed out that all that is required in order to satisfy the enablement requirement under 35 U.S.C. § 112, first paragraph, is making any transgenic animal, not the perfect transgenic animal. Any transgenic animal with any detectable level of expression of a transgene, for example SEQ ID NO:1, is all that is required for claim 7 to satisfy the enablement requirement, for it is well established that the enablement requirement is met if any use of the invention (or in this case, certain aspects of the invention) is provided (*In re Nelson*, 126 USPQ 242 (CCPA 1960); *Cross v. Iizuka*, 224 USPQ 739 (Fed. Cir. 1985)). “The enablement requirement is met if the description enables any mode of making and using the invention” (*Johns Hopkins Univ. v. CellPro, Inc.*, 47 USPQ2d 1705, 1719 (Fed. Cir. 1998), citing *Engel Indus., Inc. v. Lockformer Co.*, 20 USPQ2d 1300, 1304 (Fed. Cir. 1991)). Additionally, it has been established that a specification “need describe the invention only in such detail as to enable a person skilled in the most relevant art to make and use it” (*In re Naquin*, 158 USPQ 317, 319 (CCPA 1968); emphasis added).

Furthermore, it is important to remember that in assessing the question of whether undue experimentation would be required in order to practice the claimed invention, the key term is “undue”, not “experimentation” (*In re Angstadt and Griffin*, 190 USPQ 214 (CCPA 1976)). The large number of reports in the literature on a variety of species of transgenic animals, as detailed above, strongly argues against such a use requiring “undue experimentation”. In *In re Wands* (8 USPQ2d 1400 (Fed. Cir. 1988); “*Wands*”), the Office took the position that the applicant failed to demonstrate that the disclosed biological processes of immunization and antibody selection could reproducibly result in a useful biological product (antibodies from hybridomas) within the scope of the claims. In its decision overturning the Office’s rejection, the Federal Circuit found that Wands’ demonstration of success in four out of nine cell lines screened was sufficient to support a conclusion of enablement. The court emphasized that the need for some experimentation requiring, *e.g.*, production of the biological material followed by routine screening, was not a basis for a finding of non-enablement, stating:

Disclosure in application for the immunoassay method patent does not fail to meet enablement requirement of 35 USC 112 by requiring ‘undue experimentation’, even though production of monoclonal antibodies necessary to practice invention first requires production and screening of numerous antibody producing cells or ‘hybridomas’, since practitioners of art are prepared to screen negative hybridomas in order to find those that produce desired antibodies, since in monoclonal antibody art one ‘experiment’ is not simply screening of one hybridoma but rather is entire attempt to make desired antibody, and since record indicates that amount of effort needed to obtain desired antibodies is not excessive, in view of Applicants’ success in each attempt to produce antibody that satisfied all claim limitations.

*Wands* at 1400. Thus, the need for some experimentation does not render the claimed invention unpatentable under 35 U.S.C. § 112, first paragraph. Indeed, a considerable amount of experimentation may be permissible if such experimentation is routinely practiced in the art. *In re Angstadt and Griffin, supra; Amgen, Inc. v. Chugai Pharmaceutical Co., Ltd.*, 18 USPQ2d 1016 (Fed. Cir. 1991). Therefore, given the evidence detailed above concerning the ability of the skilled artisan to produce a variety of different species of transgenic animals with some detectable level of transgene expression with reasonable certainty, claim 7 meets the enablement requirement as it is supported by a specification that provides sufficient description to enable the skilled person to make and use the invention as claimed..

In the Final Action, the Examiner admitted that “numerous transgenic animals have been made to date”, but stated that “the experimental usefulness of the animals generated is often a problem”, and that “(t)he primary reason for this is that the phenotype remains highly unpredictable” (the Final Action at page 5). In the Response to the Final Action, Appellants respectfully pointed out whether or not any particular non-human transgenic animal is more or less useful in any particular specific application has **no bearing whatsoever** on whether a claim meets the enablement requirement under 35 U.S.C. § 112, first paragraph. **All** that is required in order for claim 7 to comply with the enablement requirement is that the skilled artisan be able to “**make**” and “**use**” non-human transgenic animals. The Examiner stated that “(f)actors as hard-to-control (*sic*) as the genetic background of the animal can mean success or failure in obtaining the **desired** expression that leads to the **expected** phenotypic outcome” (the Final Action at page 5, emphasis added). Appellants respectfully pointed out that there is **no** such **qualitative** requirement **anywhere** within 35 U.S.C. § 112, first paragraph, and **no** holding from either the Federal Circuit or the Supreme Court, that would require a particular transgene to have

a “desired” expression level or an “expected” phenotypic outcome in order to comply with 35 U.S.C. § 112, first paragraph. Furthermore, the Manual of Patent Examining Procedure (“the MPEP”) clearly states in Section 2164 that “to comply with 35 U.S.C. § 112, first paragraph, it is not necessary to ‘enable one of ordinary skill in the art to make and use a perfected, commercially viable embodiment absent a claim limitation to that effect’”, citing *CFMT, Inc. v. Yieldup Int’l Corp.*, 68 USPQ2d 1940, 1944 (Fed. Cir. 2003). Regarding non-human transgenic animals, claim 7 contains no specific “claim limitation to that effect”. Thus, all that is required in order to satisfy the enablement requirement under 35 U.S.C. § 112, first paragraph, is that the skilled artisan be able to “make” and “use” any non-human transgenic animal, not “make” and “use” the perfect non-human transgenic animal, or any specific non-human transgenic animal. The ability to generate a non-human transgenic animal having any detectable level of expression of a transgene, for example SEQ ID NO:1, is all that is required. As the skilled artisan is clearly able to “make” and “use” a variety of different species of transgenic animals, claim 7 is therefore enabled as it is supported by a specification that provides sufficient description to enable the skilled person to make and use the invention as claimed.

The Examiner went on to state that “(r)egarding the instant Application, in view of the lack of guidance provided by the specification for identifying and isolating embryonic cells which can contribute to the germ line of any non-human mammal other than the mouse, such as dogs or cows, the skilled artisan would not have had a reasonable expectation of success in generating any and all non-human transgenic animals using ES cell technology” (the Final Action at page 6). Appellants respectfully pointed out that “ES cell technology” is only one method of generating transgenic non-human animals taught in the specification as originally filed. As clearly set forth in the specification as originally filed at page 17, lines 11-24:

Any technique known in the art may be used to introduce a NHP transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to pronuclear microinjection (Hoppe, P.C. and Wagner, T.E., 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten *et al.*, 1985, Proc. Natl. Acad. Sci., USA 82:6148-6152); gene targeting in embryonic stem cells (Thompson *et al.*, 1989, Cell 56:313-321); electroporation of embryos (Lo, 1983, Mol Cell. Biol. 3:1803-1814); and sperm-mediated gene transfer (Lavitrano *et al.*, 1989, Cell 57:717-723); *etc.* For a review of such techniques, see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115:171-229, which is incorporated by reference herein in its entirety.

The specification clearly teaches that any technique known in the art can be used to produce non-human transgenic animals, not just “ES cell technology”. In fact, U.S. Patent No. 4,873,191, which issued on October 10, 1989 and was referenced in the specification as originally filed (as detailed in the above quote), enables the generation of any transgenic mammal utilizing pronuclear microinjection, and each of the referenced techniques have been successfully used by skilled artisans to produce a variety of non-human transgenic animals. Therefore, as the specification as originally filed clearly enables the skilled artisan to “make” and “use” non-human transgenic animals, claim 7 meets the enablement requirement under 35 U.S.C. § 112, first paragraph.

With regard to gene therapy, in the Second Action the Examiner stated that claim 7 is not enabled because “(t)he specification also discloses that ‘nucleotide constructs’ encoding NHP products can be used to genetically engineer host cells to express such products in vivo and that these products can be used in gene therapy” (the Second Action at page 4), but “the specification does not teach any methods or working examples that indicate an NHP nucleic acid was introduced into and expressed in a cell for therapeutic purposes”, and the “(r)levant literature teaches that since 1990, about 3500 patients have been treated via gene therapy and although some evidence of gene transfer has been seen, it has generally been inadequate for a meaningful clinical response” (the Second Action at page 5). Once again, with regard to the Examiner’s first argument, Appellants respectfully pointed out in the Response to the Second Action that this argument is not dispositive as to the question of enablement, for it has long been established that “there is no statutory requirement for the disclosure of a specific example” (*In re Gay, supra*). Once again, this argument alone cannot support an allegation that claim 7 is not enabled.

With regard to the Examiner’s second argument, it appears that the Examiner believes that claim 7 is not enabled for gene therapy because gene therapy does not always produce a “meaningful clinical response”. However, Appellants once again pointed out in the Response to the Second Action that it is well established that the enablement requirement is met if any use of the invention is provided (*In re Nelson, supra*; *Cross v. Iizuka, supra*), and “(t)he enablement requirement is met if the description enables any mode of making and using the invention” (*Johns Hopkins Univ. v. CellPro, Inc., supra*). Appellants reiterate that a specification “need describe the invention only in such detail as to enable a person skilled in the most relevant art to make and use it” (*In re Naquin, supra*; emphasis added). Appellants respectfully pointed out in the Response to the Second Action that there

are a number of reports in the literature, prior to the filing date of the present application and the priority application, concerning a variety of gene therapy vectors and successful gene therapy regimens. In fact, the Examiner herself admits that gene therapy can and has been practiced by the skilled artisan (“since 1990, about 3500 patients have been treated via gene therapy” and “some evidence of gene transfer has been seen”). Therefore, claim 7 is clearly enabled as it is supported by a specification that provides sufficient description to enable the skilled person to make and use the invention as claimed.

Furthermore, with regard to a requirement that the host cells of claim 7 be able to generate a “meaningful clinical response” in gene therapy applications, such an enablement standard conflicts with established patent law. As discussed in *In re Brana*, 34 USPQ2d 1436 (Fed. Cir. 1995; “*Brana*”), the Federal Circuit admonished the Office for confusing “the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption” (*Brana* at 1442). The Federal Circuit went on to state:

At issue in this case is an important question of the legal constraints on patent office examination practice and policy. The question is, with regard to pharmaceutical inventions, what must the applicant provide regarding the practical utility or usefulness of the invention for which patent protection is sought. This is not a new issue; it is one which we would have thought had been settled by case law years ago.

*Brana* at 1439, emphasis added. The Federal Circuit concluded:

FDA approval, however, is not a prerequisite for finding a compound useful within the meaning of the patent laws. Usefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans. Were we to require Phase II testing in order to prove utility, the associated costs would prevent many companies from obtaining patent protection on promising new inventions, thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas such as the treatment of cancer.

*Brana* at 1442-1443, citations omitted, emphasis added. Thus, based on the holding in *Brana*, claim 7 clearly meets the enablement requirement under 35 U.S.C. § 112, first paragraph.

The Examiner then once again concluded that “undue experimentation would be required of the skilled artisan to introduce and express an NHP nucleic acid into the cells of an animal” (the Second Action at page 5). However, Appellants reiterate that in assessing the question of whether undue experimentation would be required in order to practice the claimed invention, the key term is “undue”,

not “experimentation” (*In re Angstadt and Griffin, supra*). Once again, the large number of reports in the literature on a variety of gene therapy vectors, and advances in gene therapy techniques, strongly argues against such a use requiring “undue experimentation”. Furthermore, the need for some experimentation does not render the claimed invention unpatentable under 35 U.S.C. § 112, first paragraph (*In re Wands, supra*). Indeed, a considerable amount of experimentation may be permissible if such experimentation is routinely practiced in the art (*In re Angstadt and Griffin, supra*; *Amgen, Inc. v. Chugai Pharmaceutical Co., Ltd., supra*). Therefore, given the evidence detailed above concerning the ability of the skilled artisan to create gene therapy constructs that have some level of success, claim 7 meets the enablement requirement.

In the Final Action, the Examiner stated that “successes in the gene therapy art have been limited and very specific”, that “design of the vector, the method of targeting, and the host responses all remain critical factors in designing a successfully (*sic*) gene therapy protocol”, and therefore “gene therapy remains unpredictable” (the Final Action bridging pages 6 and 7). Appellants once again respectfully pointed out that whether or not any particular gene therapy vector or protocol is more or less successful in any particular specific gene therapy application has no bearing whatsoever on whether a claim meets the enablement requirement under 35 U.S.C. § 112, first paragraph. It appears that the Examiner seems to believe that claim 7 is not enabled for gene therapy because gene therapy does not always produce a specific therapeutic benefit. Appellants reiterated that there is no such qualitative requirement anywhere within 35 U.S.C. § 112, first paragraph, and no holding from either the Federal Circuit or the Supreme Court, that would require a particular gene therapy vector or protocol to produce a specific therapeutic benefit in order to comply with 35 U.S.C. § 112, first paragraph. As noted above, Section 2164 of the MPEP clearly states that “to comply with 35 U.S.C. § 112, first paragraph, it is not necessary to ‘enable one of ordinary skill in the art to make and use a perfected, commercially viable embodiment absent a claim limitation to that effect’”, citing *CFMT, Inc. v. Yieldup Int’l Corp., supra*. With regard to gene therapy, claim 7 contains no specific “claim limitation to that effect”. Thus, all that is required in order to satisfy the enablement requirement under 35 U.S.C. § 112, first paragraph, is that the skilled artisan be able to “make” and “use” any gene therapy vector or protocol, not “make” and “use” the perfect gene therapy vector or protocol, or any specific gene therapy vector or protocol. As the skilled artisan is clearly able to “make” a variety of different gene therapy vectors for “use” in a variety of gene therapy protocols or regimens, claim 7 is



therefore enabled as it is supported by a specification that provides sufficient description to enable the skilled person to make and use the invention as claimed.

Therefore, based on the evidence of record that it is well-known to skilled artisan how to make and use a variety of species of transgenic animals, as well as a variety of gene therapy vectors, the 35 U.S.C. § 112, first paragraph, rejection is improper:

As a matter of patent office practice, then, a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

*In re Marzocchi*, 169 USPQ 367, 369 (CCPA 1971), emphasis as in original. Appellants respectfully point out that, as a matter of law, it is well settled that a patent need not disclose what is well-known in the art (*In re Wands, supra*). In fact, it is preferable that what is well-known in the art be omitted from the disclosure (*Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 231 USPQ 81 (Fed. Cir. 1986)). Therefore, the full breadth of claim 7 is clearly enabled.

Finally, Appellants respectfully pointed out in the Response to the Final Action that the requirements set forth by the Examiner for compliance with the enablement requirement of 35 U.S.C. § 112, first paragraph, do not comply with the requirements set forth by the Office for compliance with 35 U.S.C. § 112, first paragraph. This is underscored by numerous patents that have been issued over the years, which were filed prior to the priority date of the present application, that specifically claim “a host cell”. As just three examples of such issued U.S. Patents, the Examiner was invited to review U.S. Patent Nos. 6,492,150 (filed on July 3, 1996), 6,498,021 (filed on May 31, 2000), and 6,500,635 (filed November 28, 1994; **Exhibits P-R**; copies of issued U.S. Patents not provided pursuant to repeated requests from the Office). As issued U.S. Patents are presumed to meet all of the requirements for patentability, including 35 U.S.C. § 112, first paragraph, Appellants submit that claim 7 must also meet the requirements of 35 U.S.C. § 112, first paragraph. While Appellants understand that each application is examined on its own merits, if it is the Examiner’s position that a claim directed to “a host cell” reads “on isolated host cells, as well as host cells in the context of a multicellular, transgenic organism and host cells intended for gene therapy”, and non-human transgenic animals and gene therapy were not enabled as of September 1, 2000 (the priority date of the present

application), since the skilled artisan could not practice non-human transgenic animals or gene therapy without “undue experimentation”, Appellants cannot understand how a claim to “a host cell” in these patents, filed prior to September 1, 2000, meets the enablement requirement under 35 U.S.C. § 112, first paragraph, while claim 7 in the present application does not. Holding Appellants to a different standard of enablement would be arbitrary and capricious, and, like other clear violations of due process, cannot stand.

For each of the foregoing reasons, Appellants submit that the rejection of claim 7 under 35 U.S.C. § 112, first paragraph, must be overruled.

## **VIII. CLAIMS APPENDIX**

The claim involved in this appeal is as follows:

7. (Previously Presented) A host cell comprising the recombinant expression vector of claim 5.

## **IX. EVIDENCE APPENDIX**

No evidence has been submitted pursuant to 37 C.F.R. §§ 1.130, 1.131, or 1.132 in the present Appeal.

**Exhibit A** was submitted with the Response to the Second Action (see **Exhibit A**).

**Exhibit B** was submitted with the Response to the Second Action (see **Exhibit B**).

**Exhibit C** was submitted with the Response to the Second Action (see **Exhibit C**).

**Exhibit D** was submitted with the Response to the Second Action (see **Exhibit D**).

**Exhibit E** was submitted with the Response to the Second Action (see **Exhibit E**).

**Exhibit F** was submitted with the Response to the Second Action (see **Exhibit F**).

**Exhibit G** was submitted with the Response to the Second Action (see **Exhibit G**).

**Exhibit H** was submitted with the Response to the Second Action (see **Exhibit H**).

**Exhibit I** was submitted with the Response to the Second Action (see **Exhibit I**).

**Exhibit J** was submitted with the Response to the Second Action (see **Exhibit J**).

**Exhibit K** was submitted with the Response to the Second Action (see **Exhibit K**).

**Exhibit L** was submitted with the Response to the Second Action (see **Exhibit L**).

**Exhibit M** was submitted with the Response to the Second Action (see **Exhibit M**).

**Exhibit N** was submitted with the Response to the Second Action (see **Exhibit N**).

**Exhibit O** was submitted with the Response to the Second Action (see **Exhibit O**).

**Exhibit P** was presented in the Response to the Final Action, but not submitted pursuant to repeated requests from the Office (see **Exhibit A**).

**Exhibit Q** was presented in the Response to the Final Action, but not submitted pursuant to repeated requests from the Office (see **Exhibit B**).

**Exhibit R** was presented in the Response to the Final Action, but not submitted pursuant to repeated requests from the Office (see **Exhibit C**).



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1: Dev Biol. 1988 Nov;130(1):285-93.

Related Articles, Links

## Regulated expression of a vitellogenin fusion gene in transgenic nematodes.

Spieth J, MacMorris M, Broverman S, Greenspoon S, Blumenthal T.

Program in Molecular, Cellular, and Developmental Biology, Indiana University, Bloomington 47405.

In *Caenorhabditis elegans* the vitellogenin genes are expressed abundantly in the adult hermaphrodite intestine, but are otherwise silent. In order to begin to understand the mechanisms by which this developmental regulation occurs, we used the transformation procedure developed for *C. elegans* by A. Fire (EMBO J., 1986, 5, 2673-2680) to obtain regulated expression of an introduced vitellogenin fusion gene. A plasmid with vit-2 upstream and coding sequences fused to coding and downstream sequences of vit-6 was injected into oocytes and stable transgenic strains were selected. We obtained seven independent strains, in which the plasmid DNA is integrated at a low copy number. All strains synthesize substantial amounts of a novel vitellogenin-like polypeptide of 155 kDa that accumulates in the intestine and pseudocoelom, but is not transported efficiently into oocytes. In two strains examined in detail the fusion gene is expressed with correct sex, tissue, and stage specificity. Thus we have demonstrated that the nematode transgenic system can give proper developmental expression of introduced genes and so can be used to identify DNA regulatory regions.

PMID: 3181632 [PubMed - indexed for MEDLINE]

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# Genetic transformation of mouse embryos by microinjection of purified DNA

(gene transfer/mice)

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Contributed by Frank H. Ruddle, September 23, 1980

**ABSTRACT** A recombinant plasmid composed of segments of herpes simplex virus and simian virus 40 viral DNA inserted into the bacterial plasmid pBR322 was microinjected into pronuclei of fertilized mouse oocytes. The embryos were implanted in the oviducts of pseudopregnant females and allowed to develop to term. DNA from newborn mice was evaluated by the Southern blotting technique for the presence of DNA homologous to the injected plasmid. Two of 78 mice in one series of injections showed clear homology, though the injected sequences had been rearranged. Band intensities from the two positive mice were consistent with the presence of donor DNA in most or all of the cells of the newborns. These results demonstrate that genes can be introduced into the mouse genome by direct insertion into the nuclei of early embryos. This technique affords the opportunity to study problems of gene regulation and cell differentiation in a mammalian system by application of recombinant DNA technology.

Introduction of specified gene sequences into mammalian embryos can be a powerful tool for the study of developmental genetic problems. The fate of such genes can be monitored throughout development by using sensitive probing techniques offered by recombinant DNA technology. In addition, the functioning of foreign genes in a normal host environment can be used to study the processes of gene regulation and to study the physiologic roles of products of such genes more precisely. Introduction of foreign DNA into all cells of an intact animal also provides an opportunity to pass sequences to offspring and to generate large numbers of transformed animals. In order to realize these benefits, it is necessary to transform embryos early in development and allow integration of foreign DNA into the cellular progenitors of the entire animal.

Such experiments with mammals are difficult. Zygotes must be maintained in culture conditions that at least grossly approximate the oviductal environment. Moreover, they can be maintained *in vitro* for only a few days, after which they must be returned to a female for implantation and further development. Insertion of material into early mammalian embryos is also difficult because of their small size.

Investigators have recently succeeded in constructing mosaic mice composed in part of descendants from cultured teratocarcinoma cells (1-3). This advance makes possible the introduction of genes into cultured cells, which might then be induced to cooperate in the formation of an intact adult mouse (4, 5). These cultured cells are often aneuploid, however, and some difficulty has been encountered in obtaining functional germ cells derived from them (6). Another problem with teratoma mosaics is that they are, indeed, mosaics. Thus, teratoma cells of XX chromosomal constitution cannot make sperm in

mice that develop as males; the possibility of germ-line transmission in this system is accordingly reduced. Jaenisch and Mintz (7) have provided evidence that whole DNA of simian virus 40 (SV40), when placed in cavities of mouse blastocysts, may be found in the resultant offspring. Ideally, however, one would like to introduce a small amount of well-defined genetic material directly into normal embryos and allow this material to integrate and function within the host genome.

We have approached this problem by injecting DNA directly into the pronuclei of fertilized mouse oocytes. The one-cell stage was chosen in order to limit as much as possible the development of mosaicism during cleavage. To avoid the hazards of culture, injected embryos were immediately implanted into the oviducts of pseudopregnant recipients. The DNA chosen for injection was the bacterial plasmid pBR322 into which had been inserted fragments of herpes simplex and SV40 viral DNA. This plasmid was constructed because the SV40 fragment is known to contain an origin of DNA replication, whereas the herpes fragment codes for a gene product, thymidine kinase (TK), distinguishable from the endogenous mouse enzyme. DNA was extracted from newborn mice and screened by the Southern blotting technique for the presence of sequences homologous to the injected plasmid. Two of 78 mice evaluated in one experimental series were found to contain such sequences. In both instances the injected DNA had been modified, but it could be demonstrated to be derived from donor material. The intensity of the positive bands indicated that an amount of DNA roughly equivalent to one copy in every cell of the newborns was retained. We thus provide evidence that mice can be genetically transformed by direct insertion of DNA into early embryos.

## MATERIALS AND METHODS

Mice. CD-1 mice were obtained from the Charles River Breeding Laboratories. B6D2F<sub>1</sub> mice were obtained from the Jackson Laboratory. All mice were maintained on a 14:10 light-dark schedule (lights off at 10 p.m., on at 8 a.m.). Six-week-old females were induced to superovulate with 5 international units of pregnant mares' serum (Cestyl, Organon) at 4 p.m. followed 48 hr later by 2.5 international units of human chorionic gonadotropin (Pregnyl, Organon) and placed immediately with males for mating. B6D2F<sub>1</sub> females were mated with CD-1 males; CD-1 females were mated with B6D2F<sub>1</sub> males. On the same evening other mature CD-1 females were placed with vasectomized CD-1 males. On the morning after mating (day 0) all female mice were examined for vaginal plugs. Six-week-old females were killed at 2 p.m. on day 0 and

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Abbreviations: SV40, simian virus 40; TK, thymidine kinase; kb, kilobase(s).

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their oviducts were removed into Krebs-Ringer bicarbonate-buffered medium supplemented with bovine serum albumin (8) and hyaluronidase at 1 mg/ml. Oviducts were opened with forceps and the fertilized eggs with remaining follicle cells were expressed into the dish. After 1–2 min, eggs were removed and washed three times in 2 ml of culture medium equilibrated with 5% CO<sub>2</sub> in air at 37°C. Eggs containing pronuclei were identified under the dissecting microscope and placed in lots of 20 in a microdrop of equilibrated medium, which was placed in a 100-mm tissue culture dish and covered with mineral oil (Mallinckrodt 6358). Eggs were stored in this manner in the incubator until microinjected.

**Microinjection.** Microneedles were pulled from thin-walled no. 1211L Omega Dot tubing (Glass Co. of America) on a DK1 model 700C pipette puller. Holding pipettes were pulled by hand on a microburner from G-12 capillary tubing (Thomas), and fire polished on a Sensar microforge. The tips of the microneedles were allowed to fill with plasmid suspension by capillary action and the barrels were then filled with Fluorinert (3M FC77). They were then secured in PE-190 intramedic tubing on a Leitz micromanipulator. Holding pipettes were also filled with Fluorinert and similarly secured in PE-90 tubing. The tubing was likewise filled with Fluorinert and attached to 1-cm<sup>3</sup> Hamilton syringes. All manipulations were carried out on a Leitz microscope.

Tissue culture dishes containing the fertilized eggs were placed on the microscope and eggs were positioned by holding the pipette such that a pronucleus near the plasma membrane was close to the microneedle. The microneedle was inserted into the pronucleus and enough plasmid suspension was injected to cause an approximate doubling of the pronuclear volume (approximately 1 pl). Eggs that survived microinjection were removed and stored in a 30-mm tissue culture dish containing 2 ml of equilibrated medium until all microinjections were completed. Injection of 40–60 embryos required 1–2 hr.

**Implantation.** Plugged pseudopregnant CD-1 females were anesthetized with Nembutal at 6 mg/100 g of body weight. Ovaries were located through a dorsal incision. The ovarian bursa was torn away with no. 5 Dumont watchmaker's forceps, taking care not to rupture large blood vessels. The ostium of the oviduct was visualized under the dissecting microscope and a pipette containing 10–20 microinjected embryos was inserted into it. The eggs were expelled into the oviduct and the wound was closed with wound clips. Mice were examined on days 18–21 for the delivery of live offspring. Newborn mice were stored at –80°C for later analysis. Sixty percent of the embryos survived microinjection; 30–50% of the survivors developed into live young. All newborns were normal in appearance. All microinjection work was carried out under P1 containment in accordance with National Institutes of Health guidelines.

**DNA Isolation.** DNA was isolated from whole newborn mice by the method of Blin and Stafford (9) with the following modifications. Powdered tissue was incubated for 4 hr at 50°C in 22 ml of 0.28 M EDTA/0.5% Sarkosyl, pH 7.0. The homogenate was subsequently extracted twice in phenol/chloroform/isoamyl alcohol (15 ml:5 ml:0.2 ml), and once in chloroform/isoamyl alcohol (15 ml:0.6 ml). The extract was dialyzed for 24 hr against 10 mM Tris-HCl, pH 8.0/10 mM NaCl/1 mM EDTA and precipitated with a 2-vol excess of 100% ethyl alcohol. Precipitated DNA was stored at –20°C until use.

**Filter Hybridization.** DNA was redissolved in 1× TEN (10 mM Tris-HCl, pH 7.75/10 mM NaCl/0.1 mM EDTA) to yield a final concentration of approximately 1 mg/ml. Twenty micrograms of DNA was digested at a 10- to 20-fold excess with appropriate restriction enzymes (Bethesda Research Laboratories, Rockville, MD). After overnight digestion at 37°C,

samples were electrophoresed in 1% agarose in 160 mM Tris-HCl/80 mM NaOAc/80 mM NaCl/5 mM EDTA, pH 8, at 350 A for 22 hr. Samples were then blotted onto nitrocellulose filters according to the method of Southern (10).

Nick translations were performed by using the New England Nuclear nick translation kit with <sup>32</sup>P-labeled dCTP obtained from New England Nuclear. Filter hybridizations were performed as described by Wahl *et al.* (11). Filters were then used to expose Kodak X-Omat x-ray film, using intensifying screens, until band intensities were appropriate for analysis.

**Construction of the Plasmid.** The recombinant plasmids, called pST6, pST9 and pST12, carrying the SV40 origin of replication and promoters, and the herpes simplex virus TK gene were constructed by inserting the SV40 *Hind*III-C fragment (12, 13) into the available *Hind*III site in the plasmid pTKX-1 (14). DNA from the SV40 mutant 1265, kindly provided by C. Cole of Yale University, was digested to completion with restriction enzymes *Hind*III and *Hinf*I (New England BioLabs) simultaneously. The double digestion generated two fragments larger than 550 base pairs; the *Hind*III-C fragment (1099 base pairs; map position 0.649–0.859) and the *Hinf*I-B fragment (1085 base pairs; 0.992–0.199), which comigrated on a 1% Seaplaque agarose gel. The 1.1-kilobase (kb) doublet band was extracted from the gel and ligated with pTKX-1 that had been digested with *Hind*III and alkaline phosphatase [as described by Ullrich *et al.* (15) except that bovine alkaline phosphatase (Sigma) was used]. The molar ratio of the vector to target in the ligation mixture was 3:1. The ligation mixture was incubated at 4°C for 17 hr with one addition of phage T4 ligase at 11 hr. The mixture was used to transform *Escherichia coli* strain HB 101, and ampicillin-resistant colonies were selected. Colonies carrying the putative pST plasmids were identified by colony hybridization (16), using SV40 DNA as the probe. Approximately 20% of the ampicillin-resistant colonies contained SV40 sequences. Confirmation of the *Hind*III-C fragment insertion and determination of its orientation in the plasmid was done by restriction analysis of mini-DNA isolations (17). A restriction endonuclease map of the plasmid pST6 is shown in Fig. 1. This work was carried out under P2 containment in accordance with National Institutes of Health guidelines.

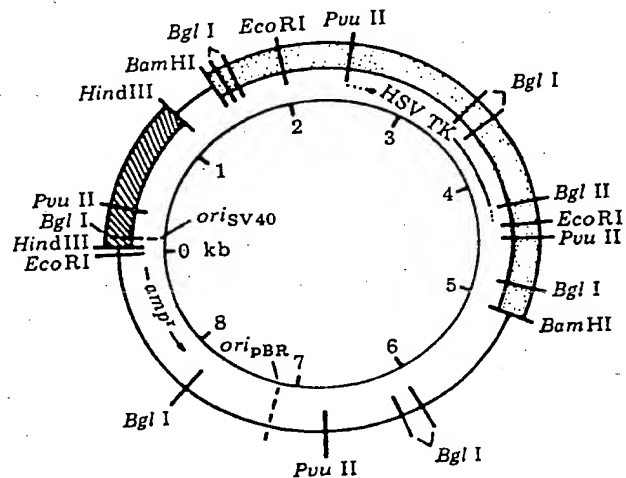


FIG. 1. The circular plasmid pST6, a derivative of pBR322. Hatched area shows the SV40 insert; stippled area denotes the herpes simplex virus TK insert. *amp*<sup>r</sup>, ampicillin resistance gene; *ori*, origin of DNA replication.

Table 1. Summary of microinjection data

Exp.	Plasmid	Copies injected per cell	Offspring	Plasmid DNA positives
1	pST6	1,000	78	2
2	pST6	12,000	10	0
3	pST6 (linearized)	1,000	40	0
4	pST9	1,000	16	0
5	pRH 1.3Mm 1	1,000	27	0
6	pST12	500	2	0
7	Uninjected control	—	54	0

The pRH 1.3Mm 1 plasmid consists of a cloned fragment of a member of the highly repeated and interspersed *EcoRI*-*Bgl* II sequence family cloned in pBR322, provided by N. Arnheim (18). pST9 is identical to pST6, except that the orientation of the SV40 fragment is reversed. pST12 is a dimer of pST6. pST was linearized by *Sal* I digestion. A total of 187 mice were born from microinjected embryos.

## RESULTS

Results of the plasmid microinjections are summarized in Table 1. In the first experimental series, injection of several hundred embryos yielded 78 live young. DNA was extracted from whole newborn mice for rapid and efficient determination of transformation frequency. The screening method gives a low estimate of the number of transformants; embryos with transforming DNA in a small percentage of their cells could have escaped detection. DNA from 2 of these 78 newborn mice contained sequences that hybridized strongly with the probe, pST6. The restriction endonuclease patterns of the incorporated sequences were significantly different between the two offspring, and are described below.

DNA from the first positive animal, no. 48, gave two intense bands with estimated sizes of 12.9 kb and 9.8 kb and a third band of very large size (>24 kb) when digested with *Bam*HI (Fig. 2). The positions of the two smaller bands were unaffected by digestion with *Hind*III, *Eco*RI, *Bam*HI, or *Xba*I (Fig. 2). This result suggested that the TK sequences, which had been inserted into the *Bam*HI sites, and the SV40 sequences inserted into the *Hind*III sites were not present in their native state in the incorporated material. The *Hind*III digestion, however, was incomplete as judged from the control track. We therefore probed with SV40 DNA alone. No sequences homologous to this

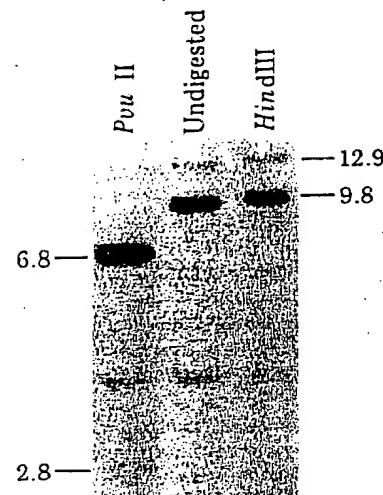


FIG. 3. DNA from mouse no. 48 digested with *Hind*III or *Pvu* II, or undigested; probed with pST6. Fragment sizes are indicated in kb.

probe were detected. The 12.9- and 9.8-kb fragments appeared in the undigested sample, consistent with their presence as free molecules. Digestion of the DNA with *Pvu* II generated two bands of altered mobility, 2.8 kb and 6.8 kb in size (Fig. 3). This result indicated that the sequences represented by the 12.9- and 9.8-kb bands contained at least one *Pvu* II site. We believe these results, taken together, are consistent with the existence of free circular molecules in the DNA of mouse no. 48.

The second positive, no. 73, showed a markedly different blotting pattern. In the undigested DNA, hybridizable material was not separable from the high molecular weight mouse DNA. Moreover, digestion with *Xba*I, which does not cut pST6, gave a single band of greater size than the highest molecular weight standard of 23.7 kb. Finally, several bands showed homology with probes synthesized from either purified SV40 DNA or TK fragment (Fig. 4). Thus, this animal had retained all or part of these portions of the plasmid.

Digestion with *Bam*HI yielded three major bands, 7.8 kb, 3.9 kb, and 3.4 kb. The largest band showed homology with

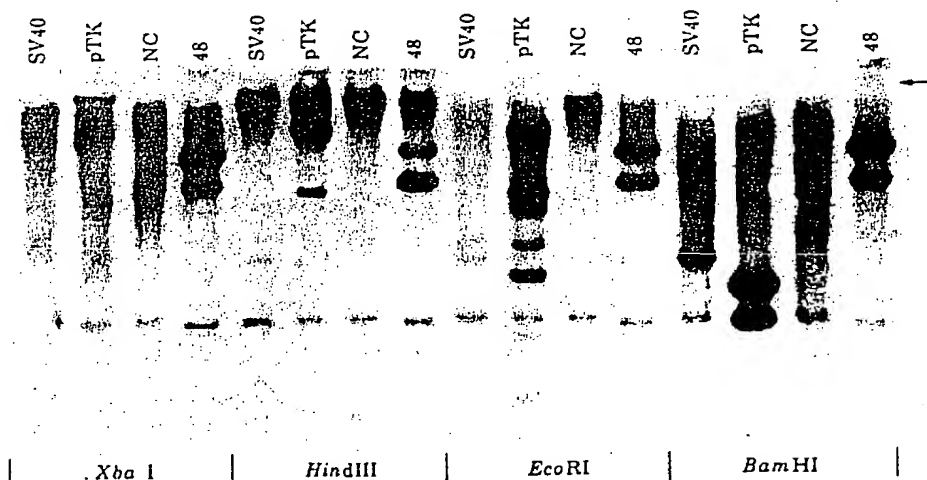


FIG. 2. DNA from mouse no. 48 digested with *Bam*HI, *Eco*RI, *Hind*III, and *Xba*I. The labeled probe was pST6 DNA. NC indicates the negative control (DNA isolated from uninjected mice). Positive controls include (i) NC DNA with SV40 DNA added (SV40) and (ii) NC DNA with the plasmid pTTX-1 added (pTK). Arrow indicates the high molecular weight band that appears reproducibly in *Bam*HI digests.



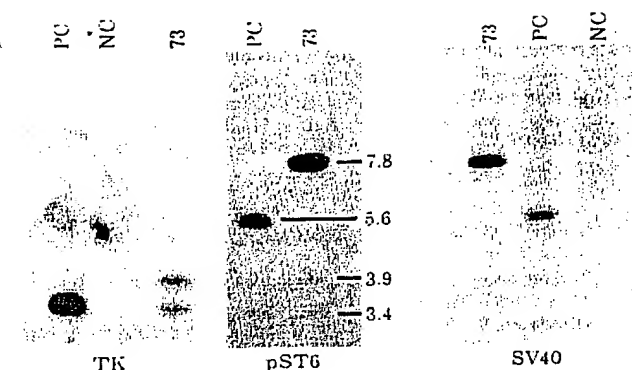


FIG. 4. DNA from mouse no. 73 digested with *Bam*HI and probed with pST6 (Center), SV40 DNA (Right), or TK fragment (Left). Positive control (PC) consists of pST6 added to mouse DNA to a concentration of  $10^{-6}$  by weight. NC denotes the negative control (DNA isolated from uninjected mice).

SV40, SV40 + pBR322, and with the whole plasmid, pST6 (Fig. 4). The two smaller bands showed homology with TK fragment, but not with SV40 or pBR322 (Fig. 4). The probeable portions of these smaller pieces were thus composed entirely of TK-derived material. The smallest band, 3.4 kb, closely approximates the size of the TK fragment that was inserted into the *Bam*HI sites, suggesting that the entire TK gene had been retained in no. 73. Digestion with *Bgl*I, however, proved this supposition incorrect. An internal fragment of TK approximately 1.8 kb in size, defined by *Bgl*I sites, did not appear in the DNA (data not shown). This showed that the 3.4- and 3.9-kb *Bam*HI fragments were composed of portions of the TK fragment that had either been concatamerized or complexed with mouse DNA to yield molecular weights equal to or greater than the molecular weight of the original TK insert.

Digests with *Pvu*II and *Hind*III provided strong evidence that the entire SV40 sequence was retained. Digestion with *Hind*III produced a fragment very close in size to the SV40 insert of pST6. In addition, digestion with *Pvu*II gave two fragments that migrated indistinguishably from the *Pvu*II-defined SV40 fragments of pST6. Thus, two independent experiments support the contention that the entire SV40 fragment was present.

## DISCUSSION

These data demonstrate that it is possible to use a recombinant plasmid as a vector for transfer of foreign genes directly into mouse embryos, and that these embryos can maintain the foreign genes throughout development. Moreover, the intensity of the bands on Southern blot analysis suggests that most or all of the cells of the newborns contained derivatives of the injected plasmid. Blotting experiments with hybrid cell populations have shown that sequences cannot be detected if present in fewer than 10% of the cells (19). We are thus confident that the two transformed mice contained enough plasmid DNA for distribution of one copy to at least this percentage of their cells. Our positive controls were adjusted to correspond to one copy of pST6 per diploid genome. The band intensities of no. 48 and no. 73 are comparable to the control. Thus, the transforming sequences are probably present in far greater amounts than the 10% threshold of detectability; the band intensities are more consistent with the presence of the plasmid derivative in most or all of the cells of the newborns. Our method of analysis cannot rule out the possibility that only a few of the cells contained all of the sequences while most of the cells were negative, but we consider unlikely the chances that cells carrying a large

amount of additional genetic material would survive and compete successfully through development. If the transforming sequences were in fact distributed throughout the tissues of the mice, then integration must have occurred at an early stage, shortly after determination of the inner cell mass. Injection of one-celled embryos may be important for obtaining early integration. In addition, the high mortality caused by microinjection suggests that injection of only a fraction of the cells of a later cleavage stage might result in preferential survival of uninjected blastomeres and consequently give a lower rate of success.

The transformation rate reported here compares very favorably with other gene transfer systems involving mammalian cells. Calcium phosphate-mediated gene transfer into cultured cells results in transformation rates of  $10^{-8}$  to  $10^{-5}$  (20, 21), while microinjection of cultured cells gives approximately 5% success (22). Our transformation rate agrees well with these latter results. The reasons for higher rates in microinjection experiments are unknown but may include the facts that DNA is inserted directly into the nucleus and that gene expression is not required in the mouse system.

Significant differences were found between the two transformed mice. In mouse no. 48, SV40 and herpes viral TK DNA could not be detected. The remaining sequences, derived from pBR322, were complexed into three bands, all of higher molecular weight than the entire pBR322 plasmid. In addition, two of these bands represented DNA that probably existed free of the host genome. The presence of unintegrated sequences in no. 48 is intriguing. Two plausible models can be invoked to explain this observation: (i) these sequences may have replicated autonomously and persisted as plasmid-like units; (ii) alternatively, they may have been generated from an integrated segment. The former model requires that the free sequences have the capacity to replicate. The plasmid from which they descended did contain the pBR322 and SV40 origins. But, interestingly, SV40 DNA is undetectable in the retained material. It is also possible that a mouse origin was acquired as a result of interaction with the host genome.

It is more likely that the free sequences were generated from integrated material. Generation of free circular DNA from transformed cultured cells has been observed previously (23). Cells infected with viruses can also generate free DNA from the integrated viral genome (24). In addition, cells transformed in calcium phosphate-mediated gene transfer experiments can pass through an unstable phase during which the donated material is maintained independent of the host genome as high molecular weight "transgenomes" (25). An important characteristic of these independent transgenomes is their rapid loss from recipient cells; as many as 10% of the cells may lose the transforming sequences per day (25). The rearrangement of the donor material in no. 48 appears analogous to transgenome formation in cultured cells. If the unintegrated sequences were similar to independent transgenomes, we would expect them to be rapidly lost from the mouse cells during development and not detectable in the newborn. The marked intensity of the two bands in no. 48 rather suggests that they were continuously being produced from an integrated sequence. The presence of a high molecular weight band after digestion with *Bam*HI is also consistent with the integration model. This band may represent material from which the two smaller bands were generated.

In mouse no. 73, no free sequences were present. Both the undigested and *Xba*I-digested samples gave single bands of greater size than the highest molecular weight standard. Moreover, SV40 and TK sequences were retained in this animal. The patterns of bands present in mouse no. 73 is explained best

by plasmid integration into the mouse genome at a site within the TK region. In this model, digestion of the mouse DNA with *Bam*HI would generate three plasmid-derived fragments, two of which would consist of the TK fragment (now at both ends of the integrated molecule) linked to mouse DNA. The third fragment would be cleaved from within the integrated plasmid and would contain the SV40 and pBR322 moieties. The predicted size of this internal fragment is 5.5 kb. This model also predicts that the TK fragment would be disrupted and that the SV40 and pBR322 sequences would be intact. The DNA of mouse no. 73 contained two bands of 3.4 and 3.9 kb that hybridized only with the purified TK fragment and contained no sequences homologous to SV40 or pBR322, and a band of 7.8 kb that hybridized to SV40 and not to TK. The large size of this fragment relative to the expected 5.5-kb fragment might be due to partial internal duplication, which is consistent with independent observations of SV40 integration (26, 27). Digestion of the DNA of mouse no. 73 with *Bgl*I or with *Pvu*II failed to generate expected fragments from within the TK insert but indicated that most or all of pBR322 and SV40 were present. Additionally, *Hind*III digestion generated a band of the expected size of the SV40 insert, indicating that all of the SV40 sequences present on pST6 were also present in the DNA of mouse no. 73 (data not shown). Thus, our observations are consistent with a single integration event.

An important similarity between the two positive mice was the extensive rearrangement of the sequences. In the first instance, SV40 and herpes virus TK sequences were largely if not entirely removed from the injected DNA. In the second case, SV40 sequences and herpes virus TK sequences were demonstrable, but the TK gene was significantly rearranged. These observations raise the possibility that selection occurred against embryos that retained the TK gene intact and in an active state. The possibility that herpes virus TK is teratogenic to mouse embryos is consistent with our data. We consider this notion unlikely, however, because cells transformed in culture and under selection for TK demonstrate similar patterns of rearrangement (25, 28).

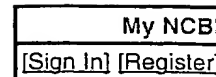
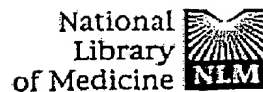
These initial results show that genetic transformation can be extended to whole mammalian organisms at a very early stage in their development. Further refinement of these techniques should lead to a reliable system of embryo transformation with its attendant applications for investigation of problems in development and cell differentiation.

**Note Added in Proof.** We have produced a third transformant by injection of 30,000 copies per cell of the plasmid pST9. Restriction analysis indicates that, as in mouse no. 73, the transforming sequences are integrated. Initial studies also indicate that at least one complete copy each of both the herpes virus TK and SV40 regions has been retained in this animal.

We thank Dr. N. Arnheim, of the State University of New York at Stony Brook, for supplying us with the pRH 1.3Mm 1 plasmid, B. Kay for advice on construction of pST plasmids, K. M. Huttner for helpful discussions, S. Pafka for photography, and M. Siniscalchi for typing this manuscript. This work was supported by National Institutes of Health Grant GM09966-19 to F.H.R., and G.A.S. was supported by National Institutes of Health Fellowship GM06528-01. J.W.G. was supported during 1979-1980 by a Hudson Brown Fellowship admin-

istered through the Department of Obstetrics-Gynecology, Yale University School of Medicine, and is currently supported by National Institutes of Health Grant IF32GM07959-01. D.J.P. was supported by Damon Runyon-Walter Winchell Cancer Fund Grant DRG 291 F and is currently supported by U.S. Public Health Service Grant 3F32 CA06414-0151. J.A.B. is supported by National Institutes of Health Training Grant 5T32-HD07149-03.

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1: Nature. 1990 Apr 5;344(6266):541-4.

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## Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene.

Mullins JJ, Peters J, Ganten D.

German Institute for High Blood Pressure Research, University of Heidelberg.

PRIMARY hypertension is a polygenic condition in which blood pressure is enigmatically elevated; it remains a leading cause of cardiovascular disease and death due to cerebral haemorrhage, cardiac failure and kidney disease. The genes for several of the proteins involved in blood pressure homeostasis have been cloned and characterized, including those of the renin-angiotensin system, which plays a central part in blood pressure control. Here we describe the introduction of the mouse Ren-2 renin gene into the genome of the rat and demonstrate that expression of this gene causes severe hypertension. These transgenic animals represent a model for hypertension in which the genetic basis for the disease is known. Further, as the transgenic animals do not overexpress active renin in the kidney and have low levels of active renin in their plasma, they also provide a new model for low-renin hypertension.

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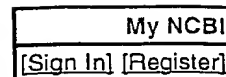
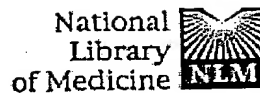
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## Production of transgenic rabbits, sheep and pigs by microinjection.

Hammer RE, Pursel VG, Rexroad CE Jr, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL.

Direct microinjection has been used to introduce foreign DNA into a number of terminally differentiated cell types as well as embryos of several species including sea urchin, *Candida elegans*, *Xenopus*, *Drosophila* and mice. Various genes have been successfully introduced into mice including constructs consisting of the mouse metallothionein-I (MT) promoter/regulator region fused to either the rat or human growth hormone (hGH) structural genes. Transgenic mice harbouring such genes commonly exhibit high, metal-inducible levels of the fusion messenger RNA in several organs, substantial quantities of the foreign growth hormone in serum and enhanced growth. In addition, the gene is stably incorporated into the germ line, making the phenotype heritable. Because of the scientific importance and potential economic value of transgenic livestock containing foreign genes, we initiated studies on large animals by microinjecting the fusion gene, MT-hGH, into the pronuclei or nuclei of eggs from superovulated rabbits, sheep and pigs. We report here integration of the gene in all three species and expression of the gene in transgenic rabbits and pigs.

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*Bio/Technology* 8, 140 - 143 (1990)  
doi:10.1038/nbt0290-140**Rabbit  $\beta$ -Casein Promoter Directs Secretion of Human Interleukin-2 into the Milk of Transgenic Rabbits**Th. A. Bühler<sup>1</sup>, Th. Bruyère<sup>2</sup>, D. F. Went<sup>1</sup>, G. Stranzinger<sup>1</sup> & K. Bürki<sup>2</sup><sup>1</sup>Swiss Federal Institute of Technology Zürich, Institute for Animal Science, CH-8092 Zürich.<sup>2</sup>Preclinical Research, Sandoz Ltd., CH-4002 Basel.

To test the potential usefulness of transgenic rabbits as production systems for human proteins of pharmaceutical value, we cloned the rabbit  $\beta$ -casein promoter and fused it to the genomic sequence of the human interleukin-2 (hIL2) gene. Four transgenic female rabbits were tested for expression and biological activity of the foreign protein in their milk. The milk of all four females proved to contain biologically active hIL2. The results show that transgenic rabbits may represent a convenient and economic system for the rapid production of biologically active protein in milk.

## ABSTRACT

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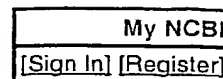
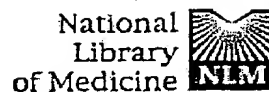
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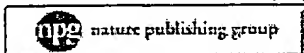
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Gentamicin administration often results in cochlear and/or vestibular hair cell loss and hearing and balance impairment. It has been demonstrated that adenovirus-mediated overexpression of glial cell line-derived neurotrophic factor (GDNF) can protect cochlear hair cells against ototoxic injury. In this study, we evaluated the protective effects of adenovirus-mediated overexpression of GDNF against gentamicin ototoxicity. An adenovirus vector expressing the human GDNF gene (Ad.GDNF) was administered into the scala vestibuli as a rescue agent at the same time as gentamicin, or as a protective agent, 7 days before gentamicin administration. Animals in the Rescue group displayed hearing thresholds that were significantly better than those measured in the Gentamicin or Ad.LacZ/Gentamicin groups. In the Protection group, Ad.GDNF afforded significant preservation of utricular hair cells. The data demonstrated protection of the inner ear structure, and rescue of the inner ear structure and function against ototoxic insults. These experiments suggest that inner ear gene therapy may be developed as a clinical tool for protecting the ear against environmentally induced insults.

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## Article



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### Spiral Ganglion Neurons Are Protected from Degeneration by GDNF Gene Therapy

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#### Abstract:

Perceptual benefits from the cochlear prosthesis are related to the quantity and quality of the patient's auditory nerve population. Multiple neurotrophic factors, such as glial cell line-derived neurotrophic factor (GDNF), have been shown to have important roles in the survival of inner ear auditory neurons, including protection of deafferented spiral ganglion cells (SGCs). In this study, GDNF gene therapy was tested for its ability to enhance survival of SGCs after aminoglycoside/diuretic-induced insult that eliminated the inner hair cells. The GDNF transgene was delivered by adenoviral vectors. Similar vectors with a reporter gene (lacZ) insert served as controls. Four or seven days after bilateral deafening, 5 ml of an adenoviral suspension (Ad-GDNF or Ad-lacZ) or an artificial perilymph was injected into the left scala tympani of guinea pigs. Animals were sacrificed 28 days after deafening and their inner ears prepared for SGC counts. Adenoviral-mediated GDNF transgene expression enhanced SGC survival in the left (viral-treated) deafened ears. This observation suggests that GDNF is one of the survival factors in the inner ear and may help maintain the auditory neurons after insult. Application of GDNF and other survival factors via gene therapy has great potential for inducing survival of auditory neurons following hair cell loss.

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**Introduction of the human growth hormone gene into the guinea pig mammary gland by in vivo transfection promotes sustained expression of human growth hormone in the milk throughout lactation.**

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We tested the feasibility of transfecting mammary tissue in vivo with an expression plasmid encoding the human growth hormone (hGH) gene, under the control of the cytomegalovirus promoter. Guinea pig mammary glands were transfected with plasmid DNA infused through the nipple canal and expression was monitored in control and transfected glands by radioimmunoassay of milk samples for hGH. Sustained expression of hGH throughout lactation was attained with a polyion transfection complex shown to be optimal for the transfection of bovine mammary cells, in vitro. However, contrary to expectations, hGH expression was consistently 5- to 10-fold higher when DEAE-dextran was used alone for transfection. Thus polyion complexes which are optimal for the transfection of cells in vitro may not be optimal in vivo. The highest concentrations of hGH in milk were obtained when glands were transfected within 3 days before parturition. This method may have application for studying the biological role or physical properties of recombinant proteins expressed in low quantities, or for investigating the regulation of gene promoters without the need to construct viral vectors or produce transgenic animals.

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## High-level synthesis of a heterologous milk protein in the mammary glands of transgenic swine

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**ABSTRACT** The whey acidic protein (WAP) is a major milk protein in mice, rats, and rabbits but has not been found in milk of livestock including swine. To determine whether mammary gland regulatory elements from the WAP gene function across species boundaries and whether it is possible to qualitatively alter milk protein composition, we introduced the mouse WAP gene into the genome of swine. Three lines of transgenic swine were analyzed, and mouse WAP was detected in milk from all lactating females at concentrations of about 1 g/liter; these levels are similar to those found in mouse milk. Expression of the corresponding RNA was specific to the mammary gland. Our results suggest that the molecular basis of mammary-specific gene expression is conserved between swine and mouse. In addition the WAP gene must share, with other milk protein genes, elements that target gene expression to the mammary gland. Mouse WAP accounted for about 3% of the total milk proteins in transgenic pigs, thus demonstrating that it is possible to produce high levels of a foreign protein in milk of farm animals.

Milk protein genes are transcribed in the mammary gland of lactating animals, and the encoded proteins are secreted in large quantities into milk. The whey acidic protein (WAP) is an abundant milk protein in mice (1, 2) but has not been found in swine or other livestock. Expression of the WAP gene is confined to the mammary gland (2, 3) and is under the control of steroid and peptide hormones as well as other developmental signals during pregnancy (4-6).

By targeting synthesis of foreign proteins to the mammary gland of transgenic animals, it should be possible to produce valuable proteins on a large scale in milk (7, 8). The combined properties of high activity and tissue-specificity make the murine WAP gene promoter a good candidate for targeting gene expression to the mammary gland. Towards this end we previously have expressed a hybrid gene containing regulatory elements from the mouse WAP gene and coding sequences from human tissue plasminogen activator in the mammary gland of transgenic mice (5, 6) and analyzed the protein in milk (5, 9). By characterizing the WAP gene, it may be possible to use its control elements to target expression of hybrid genes in farm animals. However, it is not known whether mammary regulatory elements are gene specific and whether they are functional across species boundaries. In addition, it is not known if the presence of a novel protein may adversely affect the physiology of the mammary gland. To address these questions we introduced the unmodified mouse WAP gene (10) into swine, which themselves do not contain a WAP gene, and analyzed expression of RNA and protein. With this approach, potential problems in interpreting expression data from hybrid genes would not be a factor. Also, potential deleterious physiological effects of a foreign

protein might be minimized because the target gene encodes a milk protein that would be confined primarily to the mammary gland.

Swine were chosen for these studies because they offer both economy in animal resources and time when compared to ruminantia as a transgenic animal model and because the questions being addressed did not require harvesting large quantities of milk that would be more easily obtained from dairy animals such as cows, goats, or sheep. The two primary constraints in any large animal transgenic project are the number of fertilized ova obtainable and the number of embryo recipients available. On average it is possible to recover 2-3 times more injectable ova per donor gilt than can be collected from a cow, doe, or ewe. The efficiency of producing expressing transgenic pigs or sheep per injected ovum is about 0.3% (calculated from refs. 11 and 12). Though a live-born-expressing transgenic calf has not been reported, a larger number of ova will probably be required to produce an expressing transgenic cow (13). Furthermore, because swine are polytocous, a recipient sow can carry 5 times as many fetuses as a cow, doe, or ewe. Additionally, the generation interval of swine is  $\approx$ 11 months, whereas that of goats is between 11 and 21 months and that of cattle at least 24 months. Considering all of these factors, the use of swine rather than cows, goats, or sheep requires one-sixth the number of animals, with results obtainable in less than half the time.

### MATERIALS AND METHODS

**Production of Transgenic Pigs.** Ovulation control and egg recovery were performed as described (14). Briefly, the time of ovulation of sexually mature gilts was controlled by feeding 15 mg of Altrenogest (R-2267, 17-allyl-hydroxyestra-4,9,11-trien-3-one, Roussel-Uclaf) daily for 5-9 days, beginning on day 12 and ending on day 15 of the estrous cycle. Twenty-four hours after the last feeding of Altrenogest, each gilt was given 1000 to 2000 international units of pregnant mare's serum gonadotropin (PMSG) by subcutaneous injection, and 79 hr later each gilt was given an intramuscular injection of 500 international units of human chorionic gonadotropin (hCG). Estrus behavior was monitored, and embryo donor gilts were either bred with a fertile boar or were artificially inseminated with fresh semen twice during estrus.

Approximately 58-61 hr after the hCG injection (18-21 hours after the expected time of ovulation), the reproductive tracts of donor gilts were exposed by midventral laparotomy during general anesthesia. Ova were recovered by flushing 20 ml of Dulbecco's phosphate-buffered saline (15) from the uterotubal junction through the cannulated infundibular end

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Abbreviation: WAP, whey acidic protein.

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of each oviduct. Recovered ova were immediately transferred into BMOC buffer (16) prior to microinjection and maintained at 38°C.

Pig ova are optically opaque and, as a consequence, their nuclear structures are not visible. However, centrifuging ova at  $\approx 15,000 \times g$  for 3–8 min displaces the opaque material in the cytoplasm, thereby allowing the nuclear structures to be visualized (14). Pig ova were centrifuged, and a pronucleus of one-celled ova or both nuclei of two-celled ova were injected with a TE solution (1 mM Tris-HCl/0.1 mM EDTA, pH 7.2) containing  $\approx 2$  ng of a 7.2-kilobase (kb) *EcoRI* fragment per  $\mu$ l that contained the mouse WAP gene (10). The fragment contained the entire transcribed region with its four exons, three introns, and 2.6-kb 5' and 1.6-kb 3' flanking sequences. Microinjections were performed with the aid of differential interference contrast optics at 200-fold magnification, essentially as described for mouse ova (17).

Between 20 and 30 injected ova were deposited into the ampullar region of one oviduct of each recipient gilt whose reproduction cycle had been synchronized with Altrenogest (but not superovulated—i.e., not given PMSG) or whose estrous cycle naturally coincided with the desired stage. Some recipients also received 2–4 uninjected control ova to increase the likelihood of maintaining pregnancy in the event that a majority of the microinjected eggs failed to develop. Time between microinjection and embryo transfer was about 30 min.

To identify transgenic piglets, DNA from tail biopsies was prepared and analyzed for the mouse WAP gene by Southern blotting. Offspring in the  $F_1$  generation were analyzed by the polymerase chain reaction by using primers specific to the WAP gene.

**Analysis of Mouse WAP.** Milk whey proteins were separated under denaturing conditions in sodium dodecyl sulfate (SDS)/16% polyacrylamide gels and either stained with Coomassie Blue or transferred to nitrocellulose filters. After transfer the membrane was incubated overnight in TBS (20 mM Tris-HCl, pH 7.5/500 mM NaCl) containing 3% gelatin and then was washed in TTBS (TBS containing 0.05% Tween 20). The membrane was then probed for 90 min with a 1:200 dilution of rabbit anti-WAP serum, followed by washing and incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG in TBS containing 1% bovine serum albumin for 1 hr. The antibody-antigen complexes were stained with nitrobluetetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl, pH 9.5/100 mM NaCl/5 mM  $MgCl_2$ .

**Isolation of RNA and Northern Blot Analysis.** During necropsy, tissues were immediately placed in liquid nitrogen and stored at  $-80^\circ C$ , and total RNA was isolated (18). RNA samples containing 1  $\mu$ l of ethidium bromide solution (1 mg/ml) were electrophoresed in 1.5% agarose/formaldehyde gels. The gels were blotted onto GeneScreenPlus nylon membranes, which were then probed with a randomly primed labeled 450-base-pair (bp) cDNA fragment that spanned the mouse WAP coding region.

## RESULTS

**The Mouse WAP Gene in Transgenic Swine.** Eight-hundred and fifty ova were recovered and microinjected, of which two-thirds were at the one-cell stage of development. The injected DNA contained 7.2 kb of the mouse WAP gene (see *Materials and Methods*, ref. 10). The microinjected ova along with 34 control ova were transferred into 29 recipient gilts. Twenty-two of the recipients carried their pregnancies to term, resulting in the birth of 189 pigs. DNA analysis of tail biopsies revealed that 5 (2 males and 3 females) of the piglets had incorporated the mouse WAP gene into their genomes. Approximately 1% of the injected ova resulted in transgenic

founders. From other transgenic pig projects using different gene constructs, the efficiency of producing founder pigs was similar (11). In this study one pig was stillborn and one died shortly after birth. Such deaths are not uncommon in the pig industry, where neonate mortality is in the range of 15–20%. Lines from the three surviving pigs were established, and offspring were analyzed. Male founder 1301 was bred to three nontransgenic females; 4 of 32 offspring were transgenic, suggesting that he was mosaic for the WAP gene. Transgenic mouse breeding studies have estimated that about 30% of transgenic founders are germ-line mosaics (19). Based on Southern blot analyses, this line contains  $\approx 10$  intact copies of the WAP gene in a head-to-tail arrangement at a single locus. Female founder 2202 carried  $\approx 15$  copies of the WAP gene. She was bred at 8 months of age; 4 of 9 offspring were transgenic. She was bred a second time and died of an unknown cause 4 days before anticipated parturition. The two transgenic daughters from her first litter were also bred, and after farrowing, milk and RNA were analyzed. Female founder 1302, carrying  $\approx 10$  copies of the WAP gene, was unsuccessfully bred three times. After the third failure, she was superovulated as a means of diagnosing the cause of her reproductive failures and to collect eggs if the cause did not involve ovarian disfunction. Twenty-eight ova were recovered and transferred to two recipients. From these, 20 piglets were born of which 8 were transgenic. Apparently not all of female founder 1302's eggs had been recovered because she subsequently gave birth to 9 piglets, 5 of which were transgenic.

**Secretion of Mouse WAP into Pig Milk.** Expression of the WAP transgene in transgenic pigs was evaluated by both protein and RNA analyses. Milk from female founder 2202 and her daughter 5403, from two daughters (5511 and 5701) of male founder 1301, and from female founder 1302, was analyzed for the presence of mouse WAP. Milk proteins were separated in SDS/polyacrylamide gels and either stained with Coomassie blue or blotted onto nitrocellulose membranes and analyzed with anti-mouse WAP antibodies. WAP has a molecular mass of about 14 kDa (Fig. 1A, lane 8) and, at a concentration of about 2 mg per ml, constitutes the major

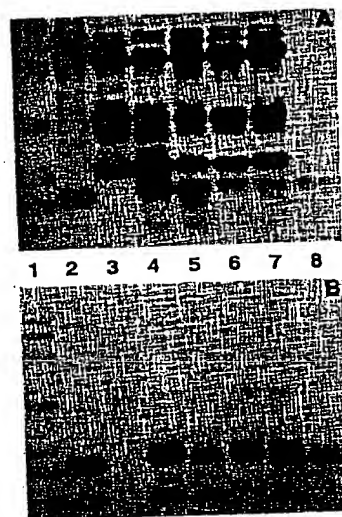


FIG. 1. Secretion of mouse WAP into milk of transgenic pigs. Milk proteins (20  $\mu$ g) were separated in SDS/polyacrylamide gels and either stained (A) or analyzed with rabbit anti-WAP antibodies (B). Lanes: 1, molecular mass markers (14, 18, 29, 45, 68, and 96 kDa); 2, total mouse whey proteins; 3–7, milk from nontransgenic pig (lane 3), pig 2202 (lane 4), pig 5403 (lane 5), pig 5701 (lane 6), and pig 5511 (lane 7); 8, 1  $\mu$ g of purified mouse WAP.

whey protein in mice (Fig. 1A, lane 2). A protein comigrating with mouse WAP was present in the milk of transgenic pigs (Fig. 1A, lanes 4–7) but not in milk from a nontransgenic control pig (Fig. 1A, lane 3). In addition, a 14-kDa protein in milk from transgenic, but not from nontransgenic, pigs reacted strongly with anti-mouse WAP antibodies (Fig. 1B). The lower molecular mass material reacting with anti-WAP antibodies probably reflects degradation products of the WAP. Taken together, this shows that the mouse WAP gene was expressed in transgenic pigs, and the encoded protein was secreted into the milk. The level of mouse WAP in the milk of each transgenic pig was determined in ELISA. By setting the level of WAP in mouse milk arbitrarily at 100%, animals 2202 and 5403 (line 2202) and animals 5701 and 5711 (line 1301) were shown to express WAP at about 100%, and female founder 1302, at about 50%. Thus, about 1–2 g of WAP was present per liter of pig milk.

WAP is secreted into mouse milk during the entire lactational period. To determine whether the expression in transgenic pigs paralleled this pattern, we analyzed WAP levels in the milk of founder female 1302 over a 4-week lactational period (Fig. 2). Whey samples were separated in SDS/polyacrylamide gels and either stained (Fig. 2A) or analyzed with anti-WAP antibodies (Fig. 2B). Constant levels of WAP were found over a 26-day period. This suggests that, at least over this period of time, the WAP transgene was coordinately regulated with other pig milk protein genes.

**Expression of Mouse WAP RNA in Pigs.** To correlate the level of WAP in milk with the corresponding RNA in mammary tissue, founder female 2202 was biopsied 11 days postpartum, and mammary RNA was analyzed with a mouse-specific WAP cDNA. An RNA of about 600 nucleotides hybridized with the WAP probe (Fig. 3, lanes b and c), confirming mouse WAP gene expression in the mammary glands of transgenic pigs. Furthermore, the RNA levels in pig 2202 and mouse were similar; this agrees with the WAP levels found in the milk. The WAP RNA in pig 2202 appeared to be

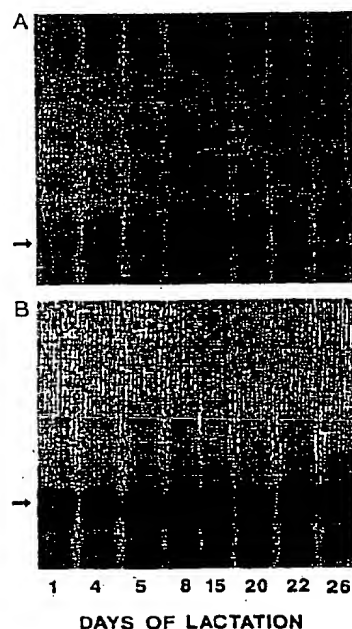


FIG. 2. Expression of mouse WAP during the lactational period of pig 1302. Milk samples were collected at various days after parturition as indicated, and whey fractions were prepared. Upon gel separation, samples were either stained (A) or analyzed with anti-WAP antibodies (B).

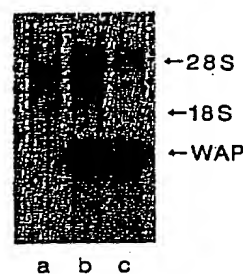


FIG. 3. Expression of mouse WAP RNA in transgenic pigs. Mammary RNAs (5  $\mu$ g) from a lactating nontransgenic pig (lane a), founder pig 2202 (lane b), and a mouse (lane c) were separated in a formaldehyde gel, transferred to a nylon membrane, and analyzed with a cloned cDNA probe specific for mouse WAP RNA.

about 10–20 nucleotides shorter than its counterpart in mice (Fig. 3). Since the protein coding region was intact, the smaller size may be due to differences in polyadenylation. RNA from a nontransgenic pig did not hybridize with the WAP probe (Fig. 3, lane a), verifying the absence of an endogenous WAP RNA in the pig mammary gland.

In lactating mice the WAP gene is expressed almost exclusively in the mammary gland with levels in nonmammary tissues at least 4 orders of magnitude lower (5). To test whether the 7.2-kb WAP transgene contained elements for stringent tissue specificity observed in mice, we analyzed tissues from lactating pigs from lines 2202 and 1301 for the presence of WAP RNA (Fig. 4). To demonstrate potential WAP expression in nonmammary tissues, we exposed the RNA blot for 24 hr (Fig. 4a and c). The specificity of WAP hybridization and the quantity of WAP RNA in the mammary gland were assessed in a 30-min exposure (Fig. 4b). In animal 5701 (line 1301), WAP RNA was only found in the mammary gland (Fig. 4c) at a level similar to that seen in a 10-day lactating mouse. The sensitivity of the assay would have

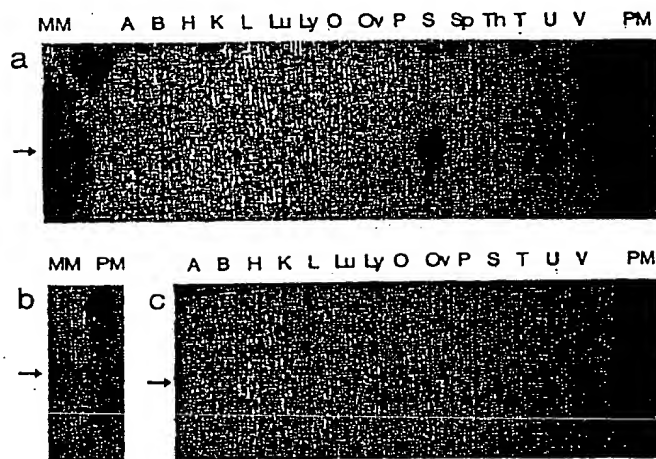


FIG. 4. Tissue distribution of WAP RNA in transgenic pigs. Pigs 5403 (a) and 5701 (c) were sacrificed, and RNA was prepared from several tissues. Upon separation in formaldehyde gels and transfer to nylon membranes, the RNA was analyzed with a probe specific for mouse WAP RNA. Lanes: MM, mouse mammary gland; PM, pig mammary gland; A, adrenals; B, brain; H, heart; K, kidney; L, liver; Lu, lung; Ly, lymph node; O, ovaries; Ov, oviduct; P, pituitary; S, salivary gland; Sp, spleen; Th, thymus; T, tongue; U, uterus; V, vulva. In a and c, 20  $\mu$ g of total RNA was loaded in lanes with the exception of mouse mammary gland (lane MM), where 4  $\mu$ g was loaded. (b) One-hour exposure of the MM and PM lanes of a. Arrows indicate the position of WAP RNA.

permitted detection of WAP RNA levels 1000-fold lower than that observed. The level of WAP RNA in animal 5403 (line 2202) was about 80% of that seen in mouse (Fig. 4a). The lower molecular mass band in the vulva RNA from animal 5701 was not reproducible and probably reflects a gel or blotting artifact. In animal 5403 WAP expression was detected in salivary gland, although at a level of only 1% of that seen in mammary tissue (Fig. 4a). Low-level expression in the salivary gland also has been described for other transgenes containing regulatory elements from milk protein genes (5, 20). Although the salivary gland and mammary gland have similar developmental patterns in that they require interaction between epithelial and mesenchymal tissue for proper duct formation to occur (21, 22), they are not considered closely related. In contrast, sebaceous glands have a common developmental origin to that of the mammary gland. However, no WAP transcripts were found in tissue taken from the vulva (Fig. 4), which is rich in sebaceous glands.

## DISCUSSION

Three lines of transgenic swine containing the mouse WAP gene have been generated and analyzed. Although swine does not contain an endogenous WAP gene, its transcription machinery recognized the mouse WAP transgene in a tissue-specific manner, and mouse WAP was secreted into milk from founder swine as well as their offspring at levels similar to those seen in mouse milk. Thus, the molecular basis for mammary-specific gene expression is conserved between swine and mouse, and it can be suggested that the mouse WAP gene shares mammary regulatory elements with pig milk protein genes.

Expression levels of the mouse WAP genes in three lines of transgenic pigs described here and in three additional lines (unpublished data), which carry between 10 and 20 copies of the transgene, were consistently high and at a level comparable to the expression level of the endogenous gene in mice. Activity of the WAP gene in pigs appears to be relatively independent of the site of integration into host chromosomes and also independent of the gene copy number. In contrast, expression of the same 7.2-kb mouse WAP gene in transgenic mice was highly dependent on the integration site of the transgene (36). It remains to be determined whether the consistently high-level expression in transgenic pigs reflects special properties of the WAP gene, such as the presence of dominant transcription elements, or whether the pig genome provides a unique permissive environment for transgene expression. A host of other transgenic swine projects (23) argues against the latter explanation. Data from the sheep  $\beta$ -lactoglobulin gene (24), the rat WAP (25) and  $\beta$ -casein (26) genes, and several hybrid genes containing mammary regulatory elements (27–30) have shown that expression was influenced by the site of integration in transgenic mice. At a minimum the present study suggests that WAP gene regulation is different in mice and swine.

This study shows that it is feasible to synthesize and secrete a heterologous milk protein in the milk of farm animals at relatively high concentrations—i.e., more than 1 g/liter. Clark and colleagues had shown that hybrid genes containing regulatory elements from the sheep  $\beta$ -lactoglobulin gene are expressed in the mammary glands of transgenic sheep (31). However, the concentrations of the encoded proteins factor IX and  $\alpha_1$ -antitrypsin were only 25  $\mu$ g/liter and 5 mg/liter, respectively (31). With another transgene, this group produced human  $\alpha_1$ -antitrypsin in mouse milk at levels of more than 1 g/liter (20). Therefore, the ability of a transgene to be expressed in the mammary gland at high levels does not appear to be related to the nature of the encoded protein (milk protein versus foreign protein) but rather to the presence of appropriate transcription elements.

We are currently testing the ability of the mouse WAP gene promoter to control expression of non-WAP structural gene sequences in pigs.

The concentration of the transgene product produced in this study should be encouraging to those who envision using the mammary gland as a bioreactor for the production of foreign proteins as an economically viable alternative to existing tissue and microbial culture systems (7, 8). Swine produce about 10 kg of milk per day (32), and, based on the expression levels discussed here, it should be possible to produce the protein of interest at a rate of about 1 kg per lactational period of 7 weeks. Since the WAP gene promoter is active in pigs during their entire lactational period, this appears to be an achievable goal, and one sow could satisfy current world's demand of blood clotting factor IX. Alternatively, to the dairy industry, the modification of the composition of milk proteins themselves may be desirable so that overexpressing heterologous or endogenous milk proteins would result in novel milk products (33).

As with other expression systems, high activity of the transgene could have adverse effects on the physiology of the mammary gland. Pigs from two lines (1301 and 2202) were unable to sustain lactation. In contrast, lactation persisted normally in female founder 1302. This animal secreted less WAP into milk than those that abrogated lactation. Agalactia has not been observed in transgenic mice that secrete into their milk heterologous milk proteins (24, 34) or pharmacologically active proteins (20, 35) at levels similar to or exceeding those described here with swine. Experiments are in progress to determine whether the premature termination of lactation exhibited by some of the pigs is associated with mammary gene expression.

**Note Added in Proof.** We have generated transgenic mice with the 7.2-kb WAP transgene described in this paper and observed that some of the animals cannot maintain lactation (T. Burdon, R.J.W., and L.H., unpublished data).

We thank Floyd Schanbacher for purified mouse WAP, Leah Schulman and Mark Spencer for technical assistance, Jim Piatt for animal care, and William Jakoby for continued support.

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## the business of xenotransplantation

past and present

In early 1996, an analyst at Salomon Brothers investment firm, detailed like never before the "unrecognised potential of xenotransplantation": a \$6 billion market in transgenic organs by 2010. The report was read by big and small investors alike--biotech venture capitalists (who pumped money into xenotransplant research), as well as newspaper personal investment columnists who featured companies like Imutran, Nextran, Alexion, and BioTransplant as "hot picks." In these heady times, some companies were even suggesting that we might each have our own Astrids, "self pigs" custom-made from our own DNA, "immunological twins" available for any spare parts we might need in the course of our lives.

Some five years later, the big profits have not yet been realized. In late 2000, a number of the original players in the xeno business reorganized their efforts for the next phase of research and development: Most notably, Novartis, the Swiss pharmaceutical giant, merged Imutran with BioTransplant to form a new company, Immerge BioTherapeutics; the new company is allied with another Novartis-funded company, Infigen, for use of Infigen's patented cloning technology. While this move was said to reflect a new commitment to xenotransplantation by Novartis, another company's "refocusing" seemed to start with a vote of no confidence for pig-to-human transplants: In August of 2000, PPL Therapeutics, a Scottish company that set out to commercialize the "Dolly" cloning technology, lost "considerable funding" for its xenotransplantation program from Geron, a California-based company that had been PPL's largest xeno backer. PPL and Geron both denied that the move should be construed as any kind of judgement on the viability of pig-to-human transplants, but PPL has had difficulty finding a new partner for its xeno program.

Here's a look at the major players in the xeno business, past and present:

### imutran

This small biotech start-up in Cambridge, England took the early lead in the race toward the organ farm: In December of 1992, at a farm in

home  
four patients  
risks  
animal welfare  
the business  
regulators

Cambridgeshire, they created "Astrid," the world's first transgenic pig, who carried human genes within her organs to help prevent rejection by the organ recipient's immune system (one of the thorniest problems facing xenotransplantation). A year and half later, Imutran announced it had produced several generations of Astrids who might be eligible for human trials by 1996. Started by a handful of scientists, the company received early funding from Sandoz pharmaceutical company whose profits were heavily derived from an immunosuppressive drug key to successful transplants. Sandoz eventually purchased Imutran outright. In 1996, Sandoz merged with Ciba-Geigy to form Novartis.

In January, 2001, Imutran completed the relocation of its xenotransplantation research to Charlestown, MA, combining with BioTransplant (another Novartis-funded company) to form Immerge BioTherapeutics. The company denies that the move was motivated by animal rights protests in the UK.

### nextran

In the early 1990's, a small biotech company in Princeton, NJ--the DNX Corporation--emerged as one of Imutran's chief rivals. Using a farm in Albany, Ohio, Nextran successfully produced transgenic pigs whose hearts survived for impressive lengths of time in baboons; they were also far along in developing pig livers as filter "bridge" organs for people awaiting transplants. In late August, 1994, the Baxter Health Care Corp. of Deerfield IL partnered with DNX to form a new company--NEXTRAN--with Baxter owning 70% of the partnership. At the time of the formation of Nextran, Baxter's biggest revenue-generator had come from its dialysis equipment, so it took a special interest in DNX, which had been developing transgenic kidneys that might one day make dialysis less necessary. In 1995, Nextran became the first to win FDA approval for human clinical trials involving transgenic pig livers.

In FRONTLINE's report, a Nextran pig saved Robert Pennington's life. It was used outside his body as a temporary "bridge" to filter Pennington's blood while he waited for a human liver transplant. Nextran also is involved in trying to solve the problem of hyperacute rejection problems facing pig-to-human transplants.

### alexion

Formed in 1992 by a group of Yale University scientists, Alexion was one of the early innovators in finding transgenic solutions to hyperacute rejection in transplant organs. Though initially focused on creating organs (their pigs were grown on farms in West Virginia and Massachusetts, Alexion has had some of its greatest success with implantation of pig nerve cells to repair spinal cord damage. In late

1998, Alexion made headlines worldwide for successfully repairing severed spinal cords in rats and monkeys using pig cells. Alexion's clinical trials continue.

### **ppl therapeutics**

Based in Edinburgh, PPL Therapeutics is licensed to commercialize the cloning technology pioneered by the Roslin Institute which surprised the world in 1997 with its creation of "Dolly," the first cloned mammal. In 1998, the company moved its xenotransplantation program to Blacksburg, West Virginia where scientists affiliated with Virginia Tech University were already involved in the research. In March of 2000, PPL's Blacksburg laboratory announced the creation of the world's first cloned pigs. (Later in the year, Wisconsin-based Infigen would be the first to clone *transgenic* pigs; these pigs were first shown nationally in FRONTLINE's report.)

In August of 2000, PPL Therapeutics' xenotransplantation program lost "considerable funding" from its major backer, Geron corporation of Northern California, who cited a change in "strategic priorities" and a desire to concentrate on stem cell work. PPL executives as well as the director of Edinburgh's Roslin Institute issued press releases denying that the Geron move was a vote of no confidence for xeno: "The institute has had a research programme on pig cloning, one application of which would be the use of pig organs for xenotransplantation. While xeno has raised a number of well-publicised issues, such as possible infection with pig viruses, these were not the basis for the decision to refocus the funding." ). PPL continued to look for partners through the Fall of 2000, but negotiations broke down, largely due to questions about the value of PPL's xeno program.

In early 2001, PPL's Blacksburg, VA lab announced that it had secured new funding--not for xeno, but for stem cell research. It's too soon to tell whether this is one company's story, or a cautionary tale for the industry.

### **biotransplant**

Founded in 1990 and taken public with a stock offering in 1996, BioTransplant was one of the early pioneers of xenotransplantation. Like their rivals, BioTransplant focused on overcoming the hyperacute rejection problem, basing their approach on the bone marrow research of Dr. David Sachs. In August, 2000, the company, which is partnered with Massachusetts General Hospital, announced a breakthrough in breeding transgenic pigs that would not transmit pig viruses, or PERV's.

In January of 2001, BioTransplant spun-off its xenotransplantation

program, partnering with Novartis (and the former Imutran) in a new company, Immerge BioTherapeutics, but keeping their offices in the Charlestown Naval Shipyard.

### **infigen**

Infigen was created in 1997 to commercialize the animal cloning techniques developed at American Breeder Service (ABS Global Inc.)--a DeForest Illinois company which is part of W.R. Grace. (ABS describes itself as "the world's leading provider of bovine reproductive services and technologies," a global marketer of dairy and beef cattle semen.) In January of 1999, Infigen and Imutran (Novartis) formed a working alliance that guaranteed Infigen's funding in exchange for use of the company's patented nuclear transfer cloning techniques.

In his FRONTLINE interview, Michael Bishop PhD Infigen's president, explains how genetically modified pigs can be created and cloned.

### **diacrin**

Founded in 1990, Diacrin became a public company in early 1996, after the FDA gave the company approval for the first-ever clinical trials of transplanted pig cells into humans. Later in 1996, Diacrin entered a joint venture with Genzyme to develop two products using pig neural cells.

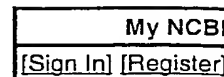
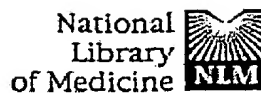
On March 16, 2001 Genzyme and Diacrin reported that a preliminary analysis of outcomes of Phase II trials for Parkinson's patients found pig neuro cell transplants did not necessarily work better than a placebo treatment. The results are likely not the end of the research trial, but the news triggered a significant drop in stock prices. Jim Finn, a Parkinson's patient featured in FRONTLINE's report, was part of a Phase I trial. Other Diacrin/Genzyme Phase I patients featured in FRONTLINE's report--Maribeth Cook and Amanda Davis--were stroke patients.

### **immerge biotherapeutics**

Beginning operations in January of 2001, Immerge BioTherapeutics is a new company formed from the UK's Imutran and the xeno division of the Boston-based BioTransplant company. Unlike the companies from which it was formed, Immerge is focused squarely on development of cells, tissues, and organs for xenotransplantation, and not on drug therapies or other transgenics.

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## Transgenic chickens: insertion of retroviral genes into the chicken germ line.

Salter DW, Smith EJ, Hughes SH, Wright SE, Crittenden LB.

We infected early chicken embryos by injection of wild-type and recombinant avian leukosis viruses into the yolk of unincubated, fertile eggs. The viremic males (designated generation 0 (G-0) were tested for transmission of proviral DNA to their G-1 progeny. Nine of 37 G-0 viremic males were mosaic and proviral DNA was transmitted to their progeny at frequencies varying from 1 to 11%. All of the G-1 progeny examined by restriction enzyme analysis for clonality of proviral junction fragments had one to three simple but different fragments. The proviral DNA was transmitted from G-1 to the G-2 progeny in a Mendelian fashion thus proving that retroviral genes have been inserted into the chicken germ line. One of the viruses is a candidate vector for insertion of foreign genes into the chicken germ line.

PMID: 3029962 [PubMed - indexed for MEDLINE]

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## Replication-Defective Vectors of Reticuloendotheliosis Virus Transduce Exogenous Genes into Somatic Stem Cells of the Unincubated Chicken Embryo

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Received 26 September 1988/Accepted 24 February 1989

Replication-defective vectors derived from reticuloendotheliosis virus were used to transduce exogenous genes into early somatic stem cells of the chicken embryo. One of these vectors transduced and expressed the chicken growth hormone coding sequence. The helper cell line, C3, was used to generate stocks of vector containing about  $10^4$  transducing units per ml. Injection of 5- to 20- $\mu$ l volumes of vector directly beneath the blastoderm of unincubated chicken embryos led to infection of somatic stem cells. Infected embryos and adults contained unrearranged integrated proviral DNAs. Embryos expressed the transduced chicken growth hormone gene and contained high levels of serum growth hormone. Blood, brain, muscle, testis, and semen contained from individuals injected as embryos contained vector DNA. Replication-defective vectors of the reticuloendotheliosis virus transduced exogenous genes into chicken embryonic stem cells in vivo.

Insertion of genetic information into the chicken provides a new in vivo approach to analyzing gene expression and its effects on avian physiology. A vector derived from Rous sarcoma virus has been used to transfer additional growth hormone genes into chicken somatic cells by infection of 7- and 9-day-old embryos (35). More recently, gene transfer into chicken germ cells (27-29) has been accomplished by infection of day-old embryos with similar replicating Rous sarcoma virus vectors (18, 33). This approach to avian gene transfer has advantages over DNA microinjection since the early chicken zygote is difficult to manipulate and even a freshly laid egg contains thousands of cells (10, 20). However, replicating retroviral vectors have disadvantages. They can result in gene transfer to susceptible cells at various stages of differentiation long after initial infection of the embryo. This can make it difficult to determine the stage of development at which gene insertion takes place or the cell lineage relationships within fully differentiated tissues. Furthermore, replicating vectors also increase the potential for disease states associated with chronic viral infection (16, 24, 38).

Replication-defective retroviral vectors offer an alternative approach (2, 6, 21, 36, 40). Such vectors, derived from reticuloendotheliosis virus type A (REV-A) (31), are produced by the helper cell line C3 which contains a packaging-defective helper provirus (40). When transfected with a defective proviral vector, this helper cell assembles infectious replication-defective vector but little or no competent virus (17). Both replicating REV and the replication-defective REV vector ME111 have been previously used for gene transfer into chicken somatic cells by injection of virus into follicles before ovulation (32). We have used a method of

gene transfer based on microinjection of vector into early embryos.

This report describes the transfer of new genetic information, including additional chicken growth hormone (cGH) coding sequences, into somatic stem cells of the chicken embryo. Chickens do not generally contain endogenous REV and express endogenous cGH only in the pituitary during late embryogenesis and after hatching (15, 19, 30). In vivo, these vectors can infect somatic stem cells of day-old chicken embryos, resulting in precociously high levels of circulating cGH and the presence of vector DNA in a variety of adult somatic tissues.

### MATERIALS AND METHODS

**Cells.** The REV-A helper cell line, C3, was generously provided by H. Temin (40). C3 cells were cultured in minimal essential medium (Eagle) containing 7% fetal calf serum—400  $\mu$ g of G418 per ml. Chicken embryo fibroblasts (CEF) were grown in F-10 medium supplemented with 10% tryptose phosphate broth—5% calf serum. D17 cells were cultured in minimum essential medium (Eagle)—7% fetal calf serum (40). Buffalo rat liver thymidine kinase (TK)-negative (BRLtk<sup>-</sup>) cells were grown in minimum essential medium (Eagle) plus 7% calf serum (39). QT-6 cells were obtained from C. Moscovici and grown as described previously (23).

**Virus infection.** BRLtk<sup>-</sup> cells were infected in medium containing 100  $\mu$ g of Polybrene per ml. CEF were infected in normal medium. Cells were usually exposed to virus overnight.

**Vectors.** The ME111 vector has been previously described (8). The vector SW272/cGH was derived by insertion of cGH cDNA downstream of the 5' long terminal repeat (LTR) of the SW272 vector (39).

**Vector assays.** TK transducing units (TKTU) released by  $5 \times 10^5$  C3 helper cells stably transfected with vector SW272/cGH were harvested after 6 h of incubation and were assayed by infection of  $10^5$  BRLtk<sup>-</sup> cells. TK-positive cells were selected for growth in medium (40) containing  $1 \times 10^{-4}$

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M hypoxanthine,  $3 \times 10^{-6}$  M thymidine, and  $5 \times 10^{-7}$  M methotrexate.

**Plasmids and chicken DNAs.** All plasmid DNAs were propagated by using derivatives of pBR322 and the HB101 strain of *Escherichia coli*. The plasmid pSW272 contains a derivative of the spleen necrosis provirus (SNV), lacks most sequences encoding the structural genes of the virus, and contains the herpes simplex virus type 1 (HSV-1) *tk* gene (40). Chicken genomic DNAs were isolated from Arbor Acres males of meat breeding lines.

**Nucleic acid isolation.** Chicken embryo DNA was prepared by solubilizing tissue in buffer containing 100 mM EDTA, 1% sodium dodecyl sulfate, 100  $\mu$ g of proteinase K per ml (pH 8). Samples were incubated at 60°C for 15 min, then at 37°C with additional protease (100  $\mu$ g/ml) for 4 h. The DNA was sheared, adjusted to 200 mM NaCl, and extracted twice with equal volumes of phenol and chloroform-isoamyl alcohol (24:1) and once with 2 volumes of chloroform-isoamyl alcohol. DNA was ethanol precipitated and dissolved in 0.01 M Tris-0.001 M EDTA (pH 8.0). Unsheared DNA was used for Southern blot analysis (34).

**Nucleic acid analysis.** DNA samples were applied to Gene Screen Plus membranes (New England Nuclear Co.) for dot blot analysis by means of 96-well plexiglass manifolds. DNA on membranes was denatured in 1.5 M NaCl-0.5 M NaOH for 15 min, neutralized in 0.5 M Tris(pH 7.5)-1.5 M NaCl for 1 min, blotted dry, and baked at 80°C for 30 min. Hybridizations were carried out as already described (17). Radiolabeled DNA probe was prepared by the method of random priming (13). Southern blot analysis was performed as described previously (34).

**cGH analysis.** cGH expression was analyzed either by radioimmunoassay (RIA) (35) or by Western immunoblotting (3).

**Transfection.** The REV-derived helper cell line, C3, was transfected as previously described (14) with the plasmids pSW272/cGH and pHyG (37). Transfected cells were selected for 10 to 14 days in medium containing 200  $\mu$ g of hygromycin per ml.

**Embryo infection.** Shell was removed from the area above the blastoderm of unincubated eggs. A Narishige micromanipulator and a 25- $\mu$ l Drummond pipette fitted with a glass needle were used to inject 5- to 20- $\mu$ l volumes of cell culture medium containing vector directly beneath the exposed blastoderm. The titer of vector was about  $10^4$  TKTU/ml as measured on BRLtk<sup>-</sup> cells. The relative titer of this vector on chicken embryo cells *in vivo* is unknown. Eggs were resealed with a patch of shell membrane which was covered with Devcon Duco cement and allowed to dry. Eggs were incubated at 37.8°C.

## RESULTS

**Vectors ME111 and SW272/cGH.** The sequence relationships among SNV, ME111, the cGH transducing vector SW272/cGH, and the packaging-defective helper proviruses present in C3 helper cells are shown in Fig. 1. ME111 has been described in detail elsewhere (8). The parental vector SW272 is derived from SNV and contains the HSV-1 *tk* gene and promoter in the same transcriptional orientation as the viral promoter (39). The cGH coding sequence was originally derived from a cDNA clone made from chicken pituitary mRNA (35). A DNA fragment *Xba*I to *Nco*I contains the complete coding sequence of the cGH gene but lacks the poly(A) addition signal present at the 3' end of the cDNA. Using Klenow reagent and blunt-end ligation, the cGH sequences were inserted into the unique *Xba*I site within

pSW272 located just downstream of the viral 5' splice donor and packaging sequence, 555 nucleotides from the 5' end of the viral RNA transcript (39). The orientation of the cGH coding sequence is the same as that of the viral sequences. Proceeding from the 5' end of the proviral RNA transcript of SW272/cGH, the first ATG encountered codes for the N-terminal methionine of cGH. SW272/cGH is designed to express cGH mRNA transcripts from the viral promoter.

**Transduction and expression of REV vectors *in vitro*.** Careful screening of the C3 helper cells transfected with pSW272/cGH and pHyG yielded clone C3-44 which released  $2 \times 10^4$  TKTU/ml into growth medium but very low levels of competent virus. Competent REV in these cultures, as estimated by infection of cultured CEF, was about 10 infectious units of REV per ml or less (17). Western blot analysis of cGH released by C3-44 cells revealed a predominantly single band of protein which comigrated with purified recombinant cGH (Fig. 2). The observed molecular size of cGH was about 23,000 daltons. The estimated concentration of cGH in a 72-h harvest of medium of clone C3-44 was at least 500 ng/ml (data not shown). CEF infected with vector released >40 ng of cGH per ml of growth medium as determined by RIA 3 days after infection (data not shown). Western blot analyses of cGH released by cell lines infected with the SW272/cGH vector are shown in Fig. 2, lanes 13 through 18. Cell lines B56 and B20 derive from the canine cell line D17. Cell lines QT82, QT54, QT15, and QT8 derive from the quail cell line QT-6. All of these cells release cGH having the same apparent molecular size as purified recombinant cGH (23 kilodaltons) (35). Approximate levels of cGH expression varied from 2 to 10 ng/ml.

**Analysis of DNA from chicken embryos after vector infection.** Tissue culture fluid (20- $\mu$ l volumes) containing the vector SW272/cGH was injected beneath the blastoderms of unincubated chicken embryos. Total embryonic DNA was isolated from vector-injected and uninjected control embryos after 7 days of development and was analyzed by qualitative dot blot hybridization with either a radiolabeled cGH probe (Fig. 3A) or a REV vector probe (Fig. 3B). The cGH probe was used to demonstrate that sufficient DNA was present on the filter for detection of vector sequences present at low copy number. Of 25 injected embryos, 13 (52%) hybridized to a radiolabeled probe of vector DNA, whereas control DNA from uninjected embryos did not.

To confirm the presence and correct genome organization of vector sequences in infected 7-day embryos, high-molecular-size DNAs from 10 vector-containing embryos were digested with *Bam*HI endonuclease and subjected to Southern blot analysis (34) (Fig. 4). The embryo DNAs examined included those from Fig. 3B, rows 1a, 2a, 6a, 7a, and 8a. Internal *Bam*HI fragments predicted from the cGH vector sequence are diagrammed in Fig. 1. Digestion of integrated proviral vector sequences of SW272/cGH should yield DNA fragments internal to the provirus of 0.86, 2.3, and 1.6 kilobase pairs (kb). A 5' junction fragment containing the 5' LTR of the vector linked to host cellular sequences adjacent to the integration site might also be detected. No 3' junction fragment containing host DNA sequences would be detected, because a *Bam*HI restriction endonuclease site is located at the 3' end of the proviral LTR. As shown in Fig. 4A, lanes 3 to 7 and 12 to 16, DNAs from these vector-infected embryos show the expected *Bam*HI DNA fragments of 0.86, 2.3, and 1.6 kb when analyzed with a probe derived from the complete SW272 plasmid DNA, which does not contain cGH sequences. The absence of detectable *Bam*HI fragments containing the junction of cellular DNA and integrated vector DNA indicates multiple sites of vector



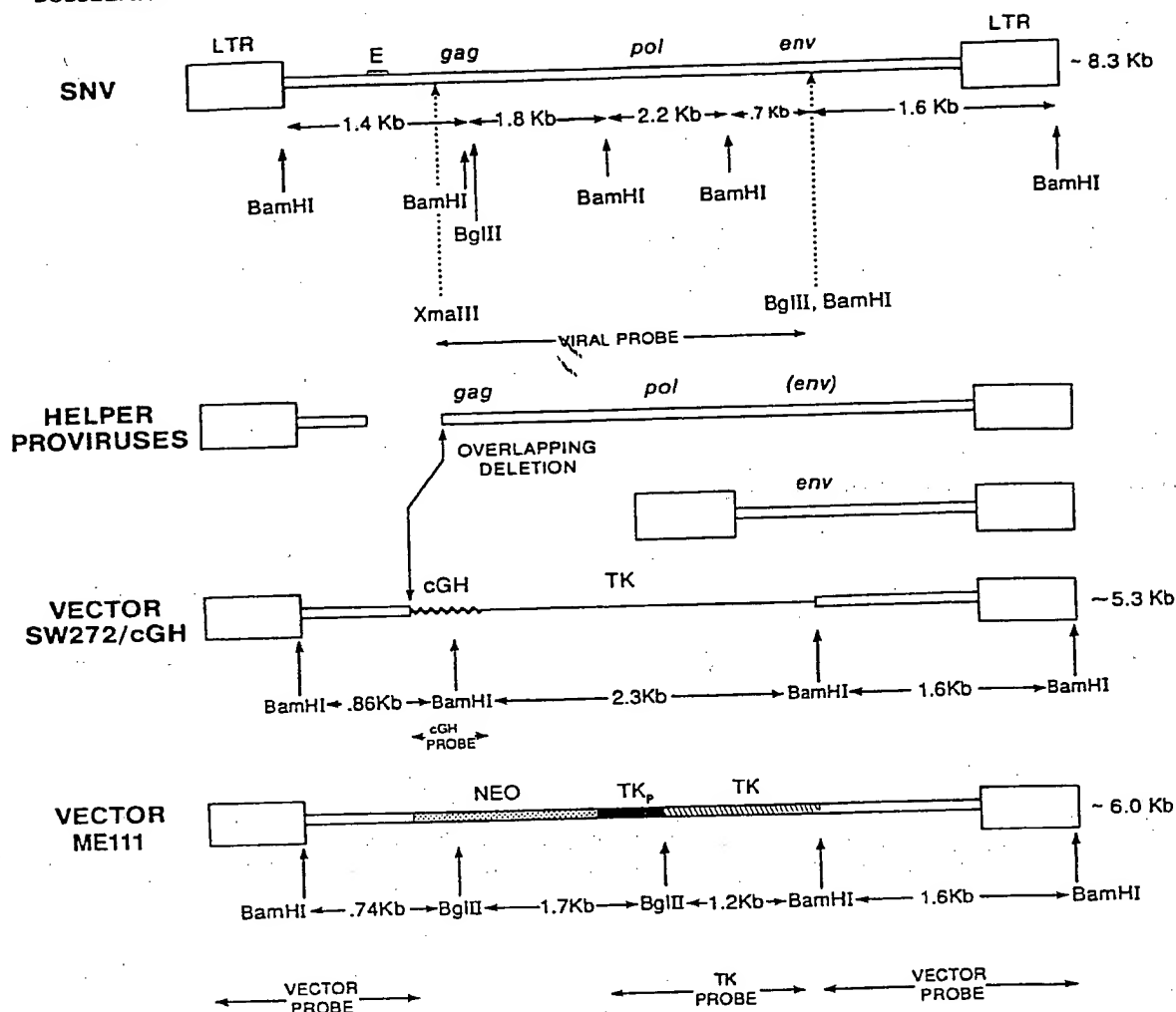


FIG. 1. Sequence relationships among the parental SNV provirus, the modified packaging-defective helper proviruses, and the vectors ME111 and SW272/cGH are shown. Relevant features of these proviruses include the LTRs, the structural genes of the virus (*gag*, *pol*, *env*), the approximate position of the packaging sequence (*E*), the cGH sequences, the HSV-1 *tk* gene promoter (TK<sub>p</sub>), the *tk* coding sequence (TK), and the neomycin phosphotransferase coding sequence (NEO). The (*env*) sequence in the larger of the two helper proviruses is presumably not expressed because of removal of the 5' splice donor. Overlapping deletions indicated between helper and vector sequences should reduce recombination between these genomes. A description of the REV helper proviruses and the original TK transducing vector from pSW272 and ME111 have been given (8, 40). The 5' LTRs of both helper proviruses derive from SNV. Their coding sequences derive from REV-A. The *env* helper provirus lacks viral splice donor and acceptor sequences. The first ATG is that of the *env* gene. The cGH vector derives from SNV. REV-A and SNV share high sequence homology. Relative sizes (in kilobases) of *Bam*HI restriction endonuclease fragments are indicated. Also given are the locations of viral, vector, TK, and cGH DNA probes.

provirus integration during infection of early embryonic cells. No 0.57-kb *Bam*HI fragment predicted from the structure of unintegrated circular forms of either the vector DNA or helper virus DNA was observed. No 1.4-kb fragment diagnostic of the 5' end of integrated replication-competent proviral SNV DNA was observed (39) (see Fig. 1). *Bam*HI-digested DNA from uninjected whole embryos or from blood of uninjected chickens did not hybridize to the vector probe (Fig. 4A, lanes 2, 8, 11, and 17, respectively).

After removal of the SW272 probe (Fig. 4B), the same filters were hybridized with a viral probe specific for the structural genes of REV to detect the presence of replication-competent virus (Fig. 4C). The parental SNV and REV-A proviruses used to derive the helper cell and vectors described here contain internal *Bam*HI fragments of 1.4,

2.2, 0.7, and 1.6 kb (see Fig. 1). Only the 1.6-kb fragment would not be detected by the virus-specific probe (Fig. 1) used in this analysis. No virus-specific *Bam*HI fragments were observed, indicating that endogenous and exogenous REV sequences were not detectable (Fig. 4C). Although this result does not rule out the presence of competent helper virus, it shows that efficient gene transfer takes place via the replication-defective SW272/cGH vector. The dot blot on the right of panel C contains various quantities of plasmid pSW253 which carries the entire REV provirus (5).

The filters shown in Fig. 4C were washed to remove probe (Fig. 4D) and were reanalyzed with a cGH-specific probe (Fig. 4E). The fragments of 0.86 and 2.3 kb in lanes 3 to 7 and 12 to 16 are the predicted cGH-containing vector sequences described in Fig. 1. The two bands (asterisks) of approxi-

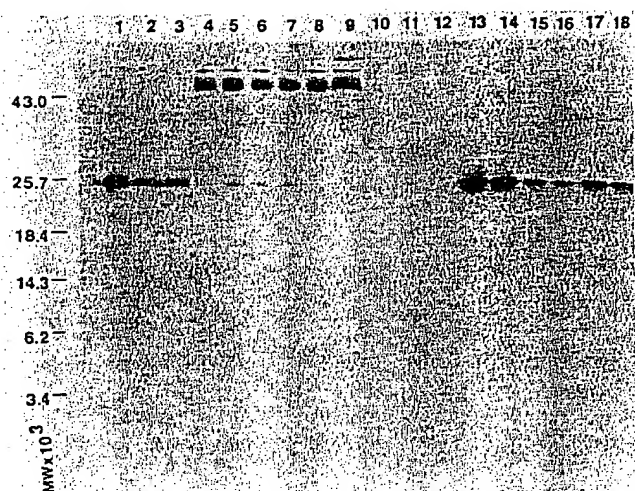


FIG. 2. Western blot analysis of cGH. Lanes: 1 to 3, 250, 125, and 30 ng per lane, respectively, of purified recombinant cGH; 4, 10  $\mu$ l of conditioned QT-6 medium; 5 to 9, immunoprecipitated cGH from various volumes of serum from 15-day vector-injected embryos, birds 18 (80  $\mu$ l), 2 (100  $\mu$ l), 6 (60  $\mu$ l), 30 (100  $\mu$ l), and 27 (200  $\mu$ l), respectively (see Table 1); 10 to 12, sample buffer alone; 13 to 18, 10  $\mu$ l of conditioned medium from clones of SW272/cGH-infected D17 and QT-6 cells (clones B56, B20, QT82, QT54, QT15, and QT8, respectively). Molecular weights (MW  $\times 10^3$ ) are shown at the left.

mately 6.4 kb and approximately 2.7 kb, which are common to all lanes, represent *Bam*HI fragments derived from the endogenous cGH gene. As expected, embryo DNAs in lanes 3 to 7 and 12 to 16 contain all four fragments derived from both the vector and endogenous gene. The 1.6-kb *Bam*HI fragment present in lanes 3 to 7 and 12 to 16 of Fig. 4A is missing in Fig. 4E, because this fragment does not contain cGH sequences.

Dot blot hybridization of DNA from brain, liver, and muscle of four 14-day embryos infected before incubation showed that two of the four embryos contained vector-specific sequences in all three tissues. One embryo contained vector sequences in liver and muscle only, and one embryo was negative (Fig. 3).

**Analysis of serum cGH.** Circulating levels of cGH were determined by RIA of serum from thirty 15-day-old embryos infected with vector before incubation (Table 1). Concentrations of cGH in serum from 16 of 30 injected embryos (55%) were at least 10 times the level in uninjected control embryos, and they ranged from 18 to 254 ng/ml. All 35 control embryos contained less than 2 ng of detectable serum cGH per ml. Western blot analysis of cGH immunoprecipitated from serum of a number of these embryos is shown in Fig. 2, lanes 5 to 9. The amount of cGH present in serum from infected embryos is similar to the amount of cGH produced in vitro by infected culture cells.

**Vector sequences in adult chickens.** Southern blot analysis of DNA isolated from blood, brain, muscle, and testis of an adult chicken (no. 87725) which had been injected as an embryo with the ME111 vector is shown in Fig. 5. DNAs were digested with *Bam*HI and *Bgl*II before analysis. The four different probes used hybridized with the REV sequence present in the vector, HSV-1 *tk* sequences of the vector, REV structural gene sequences (absent from the vector), or endogenous cGH genes. All analyzed DNAs from bird 87725 contained the predicted DNA fragments of 0.74

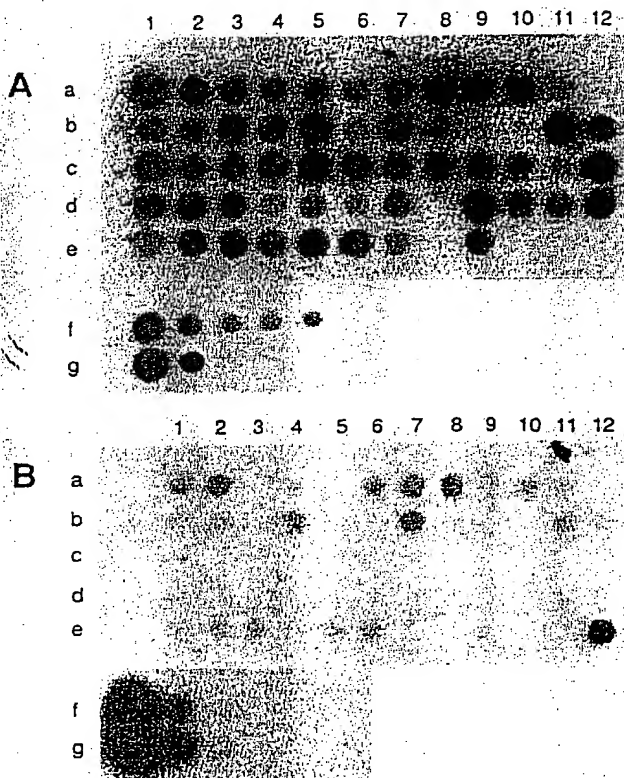


FIG. 3. Dot blot hybridization analysis of chicken embryo DNA. (A) Chicken embryo DNA hybridized with a cGH probe. Rows: a, b, and c1, total DNA from 7-day embryos injected with 20  $\mu$ l of vector C3-44; c2 to c12 and d1 to d7 and d9, DNA from uninjected control embryos; d10 to d12, e1 to e3, e4 to e6 and e7 to e9, DNA from brain, liver, and muscle of four 14-day embryos injected with vector SW272/cGH; f1 to f4 chicken blood DNA ( $\sim 15 \mu$ g) mixed with 1/10 dilutions of vector DNA starting with 1 ng in spot f1; f5, chicken blood DNA only; g1 to g4, yeast tRNA (5  $\mu$ g) mixed with the same amounts of vector DNA present in rows f1 to f4; g5, yeast tRNA only. (B) Same as in panel A, except filters were hybridized with a vector-specific probe (see Fig. 1). Row e12 is the same as row a1. Approximately 15 to 30  $\mu$ g of total embryo DNA was applied to each spot, using a 96-well blotting apparatus.

and 1.6 kb recognized by the REV vector probe and fragments of 1.2 and 1.7 kb recognized by the *tk* probe (Fig. 5A and C, respectively). DNA from blood and brain contained additional hybridizing fragments which probably include junctions between vector and cellular DNA at sites of integration (Fig. 5A, lanes 2 and 3). No REV-specific bands were observed in any of these tissue DNAs (Fig. 5B). Hybridization with cGH probe revealed endogenous fragments of  $\sim 2.7$  and  $\sim 6.4$  kb (Fig. 5D). D17 cells cocultivated with blood taken from bird 87725 at 4 weeks of age were reverse transcriptase-negative after 4 weeks of culture and did not produce detectable *tk* gene-transducing activity. Of 14 similarly derived birds, 2 were virus positive as determined by the same assay (17). Although the presence of low levels of replicating REV in birds like no. 87725 cannot be ruled out, these results are consistent with infection of embryonic stem cells with nonreplicating REV vectors.

Southern blot analysis of DNA from semen and blood of SW272/cGH-positive and control birds is shown in Fig. 6. Filters containing *Bam*HI-digested DNAs were hybridized

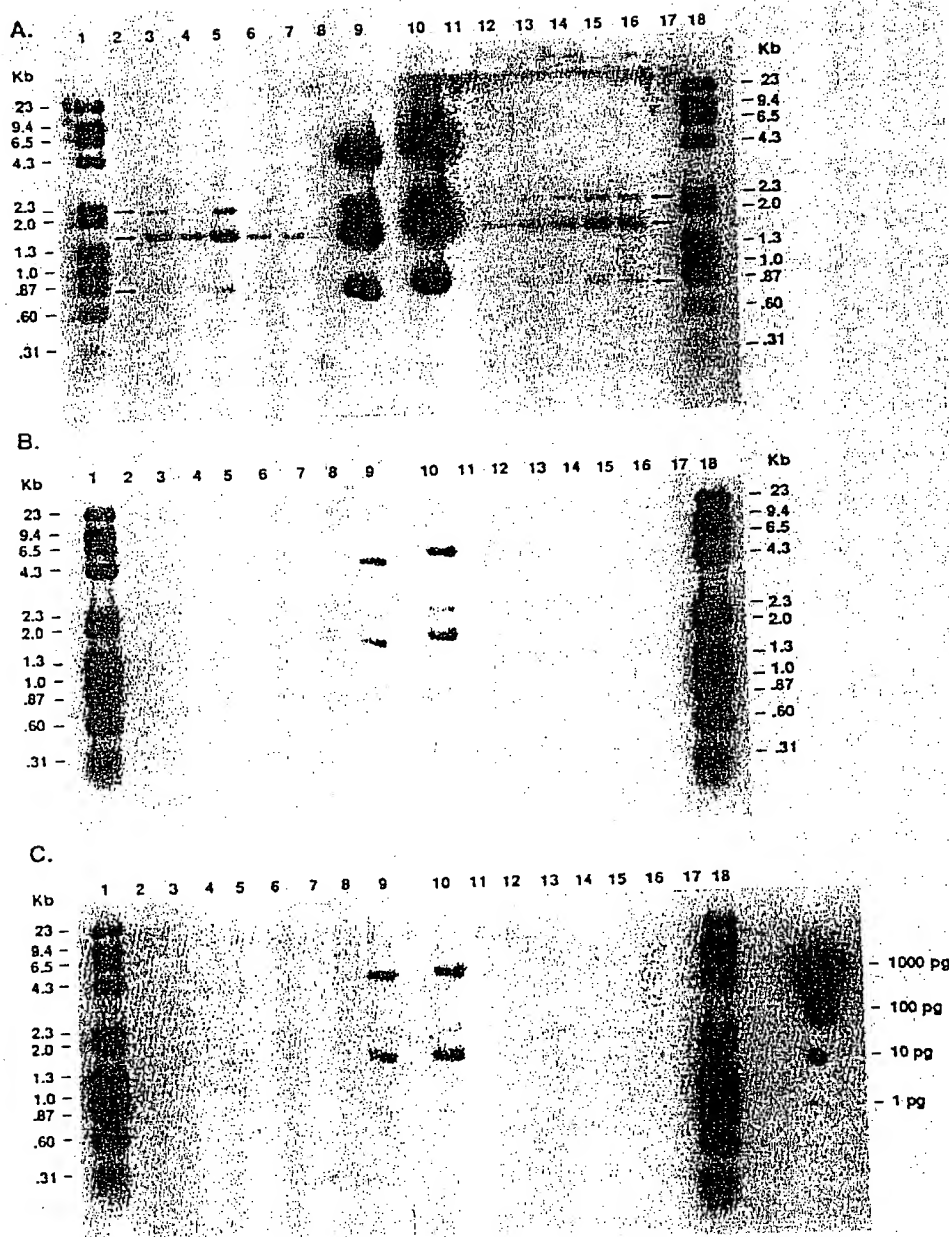


FIG. 4. Southern blot analysis of DNA from 7-day chicken embryos injected with 20  $\mu$ l of SW272/cGH vector before incubation. High-molecular-size DNA (15  $\mu$ g) was digested with *Bam*HI before analysis. The same filter was hybridized to three different probes: pSW272 probe (A), probe removed from panel A (B), virus-specific probe (C), probe removed from panel C (D), and cGH-specific probe (E). Probe hybridized to vector DNA in lanes 9 and 10 of panel A could not be completely removed. Sequences recognized by these probes are illustrated in Fig. 1. Lanes: 1 and 18, *Hind*III-digested lambda phage DNA, *Hae*III-digested  $\phi$ X174 DNA, and *Bam*HI-digested uninjected chicken blood DNA; 2 and 11, DNA from uninjected embryos; 8 and 17, DNA from blood of uninjected chickens; 3 to 7 and 12 to 16, DNA from vector-injected embryos; 9 and 10, *Bam*HI-digested DNA of pSW272/cGH (1 ng) plus uninjected chicken blood DNA. *Bam*HI fragments internal to the proviral vector are marked with arrows in panel A. *Bam*HI fragments containing the endogenous cGH sequence are marked by asterisks in panel E. Dot blot on the right of panel C contains the indicated amounts of pSW253 containing the REV-A provirus (5). Sizes are shown in kilobase pairs (Kb).

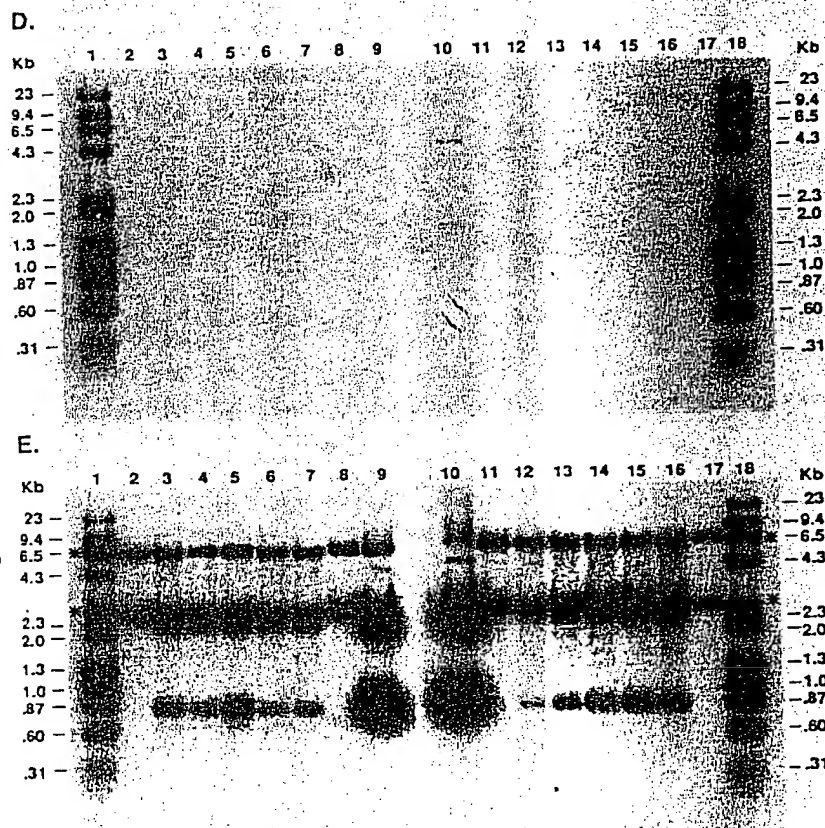


FIG. 4—Continued.

with radiolabeled DNA probes for the cGH coding sequence, 5' and 3' vector-specific sequences, or REV virus probe (see Fig. 1). Lane 1 of each panel contains a mixture of bacteriophage lambda and  $\phi$ X174 DNAs digested with *Hind*III and *Hae*III, respectively, and *Bam*HI-digested control chicken blood DNA. Lane 9 in Fig. 6A to C contains *Bam*HI-digested control chicken blood DNA and plasmid DNA of pSW272/cGH. Internal *Bam*HI fragments of vector DNA are indicated by arrows. *Bam*HI fragments derived from the endogenous cGH gene are shown by the asterisks. Internal fragments derived from the complete REV provirus are marked by chevrons. Results obtained by hybridization with the cGH-specific probe are shown in Fig. 6A. *Bam*HI-digested DNAs from control semen and blood (lanes 3 and 6, respectively) contain endogenous fragments of ~2.7 and ~6.4 kb. In lanes 4 and 7, DNAs from the semen and blood, respectively, of the vector-positive male contain an additional 0.86-kb fragment which derives from the vector SW272/cGH and hybridizes to the cGH probe. Although visible in the original autoradiogram, the 2.3-kb *Bam*HI fragment derived from the vector is not well resolved from the strongly hybridizing 2.7-kb *Bam*HI fragment derived from the endogenous cGH gene. Blood DNA appears to contain much less of the 0.86-kb fragment than does semen DNA.

Panel B of Fig. 6 shows results obtained when a similar filter was hybridized with the vector probe. Lanes 4 and 7 show that semen and blood DNA from an infected male

contain *Bam*HI fragments of 0.86 and 1.6 kb. These fragments derive from the 5' and 3' ends of the integrated vector DNA, respectively. The additional 2.3-kb internal *Bam*HI fragment of vector DNA containing HSV-1 *tk* sequences does not hybridize to the vector probe used in Fig. 6B nor does *Bam*HI-digested DNA from semen and blood of uninfected control birds (lanes 3 and 6). No 1.4-kb fragment characteristic of replicating REV was observed. The patterns of semen and blood *Bam*HI DNA fragments hybridizing with these probes are similar to each other and are consistent with the pattern observed in *Bam*HI-digested DNA from infected embryos (Fig. 4).

Panel C of Fig. 6 shows results of hybridization with a virus-specific probe. Lanes 1 to 9 are as described for panels A and B. Lane 10 is blank. Lanes 11 and 12 contain *Bam*HI-digested plasmids pSW279 (39) and pSW253 (5), respectively. DNA in lane 11 has a 5' LTR derived from SNV (with a *Bam*HI site) but a 3' LTR derived from REV-A (without a *Bam*HI site). This provirus also lacks a 310-base-pair packaging sequence (E) located near the 5' end of the provirus. The expected fragments generated by *Bam*HI digestion of this DNA are present at ~1.1 (E<sup>-</sup>), ~1.8, ~2.2, and ~0.7 kb (visible at longer exposure times). Plasmid pSW253 in lane 12 contains the REV-A provirus and lacks the *Bam*HI site present in the SNV LTR. *Bam*HI digestion of this DNA generates the observed ~1.8- and ~2.2-kb fragments. The large fragment of ~9 kb in lane 12 contains 5' and 3' portions of the provirus and a portion of the *gag* gene

TABLE 1. cGH levels in chicken embryo serum<sup>a</sup>

C3-44-injected embryos		Uninjected embryos	
Bird no.	Amt (ng/ml) of cGH	Bird no.	Amt (ng/ml) of cGH
1	51	31	<0.80
2	180	32	<0.80
3	100	33	<0.80
4	0.9	34	<0.80
5	41	35	<0.80
6	200	36	0.85
7	2.6	37	<0.80
8	80	38	<0.80
9	44	39	<0.80
10	106	40	<0.80
11	4.5	41	<0.80
12	18	42	1.2
13	8.6	43	1.0
14	1.1	44	<0.8
15	0.92	45	<0.8
16	2.2	46	<0.8
17	0.8	47	<0.8
18	254	48	<0.8
19	10.8	49	1.1
20	240	50	1.2
21	168	51	1.2
22	32	52	0.9
23	56	53	1.2
24	12	54	0.9
25	42	55	<0.8
26	0.86	56	<0.8
27	0.70	57	<1.1
28	3.4	58	<0.8
29	1.4	59	<0.8
30	0.76	60	<0.8
		61	<0.8
		62	<0.8
		63	<0.8
		64	<0.8
		65	<0.8

<sup>a</sup> Embryos of unincubated eggs were injected with 10  $\mu$ l of medium from cultures of clone C3-44. After 15 days of incubation, serum from each embryo was assayed by RIA for cGH.

sequence. The 0.7-kb fragment is observed at longer exposure times. DNAs in lanes 3, 4, 6, 7, and 9 do not contain sequences detectable with probe derived from the structural genes of REV.

## DISCUSSION

**Early chicken embryo development.** Fertilization and the first 24 h of chicken embryonic development occur in the oviduct and uterus, concomitant with the accretion of albumen and deposition of the eggshell. During this period, attempts at gene transfer into the embryo must allow for surgical removal after fertilization and either reintroduction to the oviduct or extensive artificial culture (25, 26). Both of these approaches are technically difficult. Alternatively, infection of the embryo just after oviposition represents a strategy well suited to vector-mediated gene transfer. The embryo at this stage is composed of at least 10,000 cells arranged in a disk-shaped blastoderm, one to two cells thick and 2 to 3 mm in diameter (10, 20). The day-old blastoderm floats on the yolk above a fluid-filled subgerminal cavity.

Previous studies have provided a detailed description of early chicken embryo development (10, 20) and insights regarding the developmental potential of cells comprising the embryonic blastoderm of a freshly laid egg (9, 11, 12, 22).

Separated posterior and anterior portions of very young unincubated blastoderms appear totipotent, with similar ability to form embryos in vitro (10). The slightly older blastoderm exhibits cells of both upper epiblastic and lower hypoblastic layers. Separated from the lower layer of cells, the upper epiblastic layer retains its pluripotency, regenerates a new hypoblastic layer, and can subsequently form an early-stage embryo in vitro. When dissociated and grown in culture, epiblast cells form structures resembling embryoid bodies formed by murine teratocarcinomas (22). The hypoblastic layer, in contrast, survives but does not form embryonic structures (22).

All of the above observations suggest that successful infection of the early blastoderm with REV vectors might result in gene transfer into pluripotent embryonic stem cells. However, the REVs are primarily exogenous viruses in the chicken (41). Even though infected dams can transmit the virus vertically to their offspring by shedding virus into the egg (42), nucleic acid sequences closely related to REV are not endogenous to the chicken genome (Fig. 4C). The biology of virus-host interactions may preclude stable insertion of REV sequences into the chicken genome under natural conditions. Insertion of complete REV proviruses into the chicken genome could adversely affect viability, but even defective proviruses appear to be absent from the chickens analyzed in this study.

**Infection of unincubated chicken embryo blastoderms.** We have used replication-defective REV vectors ME111 and SW272/cGH to test the feasibility of retrovirus-mediated gene transfer in the chicken. The C3 helper cell line has been used to generate titers of about  $10^4$  infectious units per ml. The ME111 vector carries the Tn5 neomycin phosphotransferase gene and the HSV-1 *tk* gene and has been described previously (8). The vector SW272/cGH carries a cDNA sequence encoding the cGH mRNA and the HSV-1 *tk* gene (see Fig. 1). Clone C3-44 released about  $10^4$  TKTU/ml and expressed about 500 ng of cGH per ml of culture medium. Analysis by RIA (35) (data not shown) and Western blotting (3) (Fig. 2) showed that cGH released by C3-44 and transduced by SW272/cGH is similar to natural cGH.

Glass needles (40 to 60  $\mu$ m diameter) were used to deposit medium containing vector directly above and below the surface of the unincubated embryonic blastoderm. This method resulted in successful transduction of vector sequences into recipient embryos. Estimates of the amount of vector injected into the space beneath the blastoderm are based on the titer on BRLtk<sup>-</sup> cells ( $\sim 10^4$  TKTU/ml) and the observation that REV titer on chicken cells could be 10- to 100-fold higher (39). We estimate that between  $10^3$  and  $10^4$  TKTU were injected per embryo.

**Vector DNA present in 7-day embryos.** Dot blot analysis of 7-day embryo DNA shown in Fig. 3 indicated that about 50% of injected embryos contained detectable vector sequences. Three different radiolabeled probes were used in Southern blot analysis of high-molecular-mass DNA from 10 embryos to distinguish the REV structural genes, the SW272/cGH vector, and endogenous cGH sequences from each other (see Fig. 1). Since most infected embryonic cells are likely to have a single copy of vector, these blots indicate that a significant percentage of the embryonic cells may carry vector sequences 7 days after infection. This is most evident in comparisons of endogenous and vector-specific *Bam*HI fragments hybridizing to the cGH probe (Fig. 4E). The lack of *Bam*HI fragments specific for replicating REV (Fig. 4C) confirms that gene transfer is primarily the result of the replication-defective REV vector and not of contaminating helper virus (17). These results show that early embryonic

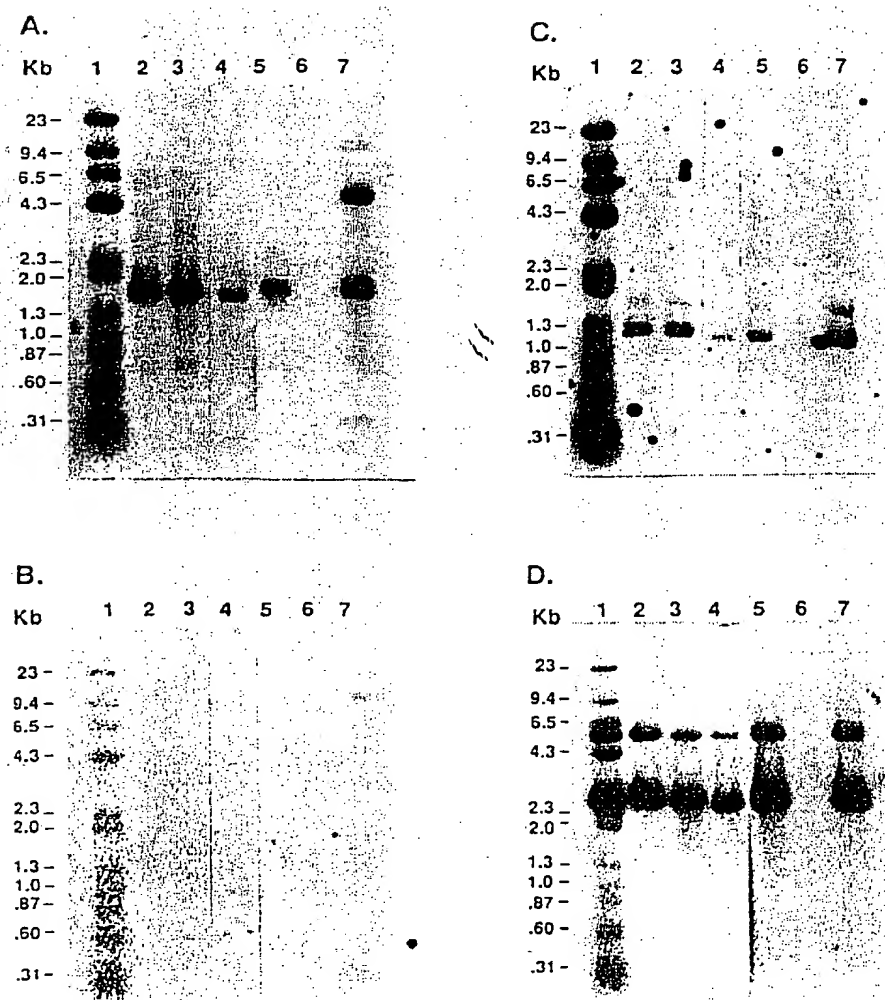


FIG. 5. Southern blot analysis of ~20  $\mu$ g of *Bam*HI- and *Bgl*II-digested DNA from tissues of ME111-positive adult male 87725. (A through D) Replicate blots hybridized with radiolabeled vector probe (A), virus probe (B), *tk* probe (C), and cGH probe (D). Lanes: 1. *Hind*III-digested lambda phage DNA, *Hae*III-digested  $\phi$ X174 DNA and *Bam*HI- and *Bgl*II-digested negative control chicken blood DNA; 2. blood DNA; 3. brain DNA; 4. muscle DNA; 5. testis DNA; 6. blank; 7. *Bam*HI- and *Bgl*II-digested pME111 (50 pg) and negative control chicken blood DNA. Sizes (in kilobases) are shown at the left of each panel.

cells are susceptible to REV infection and that they persist during development, comprising a significant fraction of the 7-day embryo.

**Expression of cGH in vector-injected embryos.** Expression of the endogenous cGH gene occurs late during embryonic development. Caudal cells of the pituitary do not contain immunodetectable cGH until day 12 of embryonic development (19), whereas detectable plasma cGH does not appear until day 17 of incubation (15). Furthermore, response of the pituitary to the cGH secretagogue, thyrotrophin-releasing hormone, is not seen until hatching (7). The absence of endogenous REV and the restricted location and timing of endogenous cGH expression facilitate the distinctions between endogenous and vector-encoded genes and their products.

Expression of the cGH gene in vivo resulted in elevated serum cGH levels in about 50% of injected embryos when measured after 15 days of development (Table 1). Levels of serum cGH in 30 injected embryos varied from <1 ng/ml to 254 ng/ml, whereas none of the 35 uninjected controls had

serum cGH levels above 2 ng/ml. Immunoprecipitated serum cGH from infected embryos comigrated with purified recombinant cGH as shown by Western blot analysis (Fig. 2). The relative contribution of somatic tissues to circulating levels of cGH is not known. These results are consistent with infection of embryonic stem cells present in the blastoderm at the time of vector injection and expression of vector-encoded cGH.

**Vector DNA in tissues of adult males.** Southern blot analysis of DNA from an adult male injected as an embryo with ME111 demonstrated the presence of vector in blood, brain, muscle, and testes (Fig. 5). Analysis of semen DNA by Southern blotting confirmed the presence of integrated unrearranged vector sequences in a low percentage of the sperm cells from a bird injected with SW272/cGH (Fig. 6). The pattern of *Bam*HI restriction fragments observed (0.86 and 1.6 kb) is consistent with that seen in Southern blot analysis of DNA from infected embryos. Probe containing HSV-1 *tk* sequences revealed the additional 2.3-kb *Bam*HI



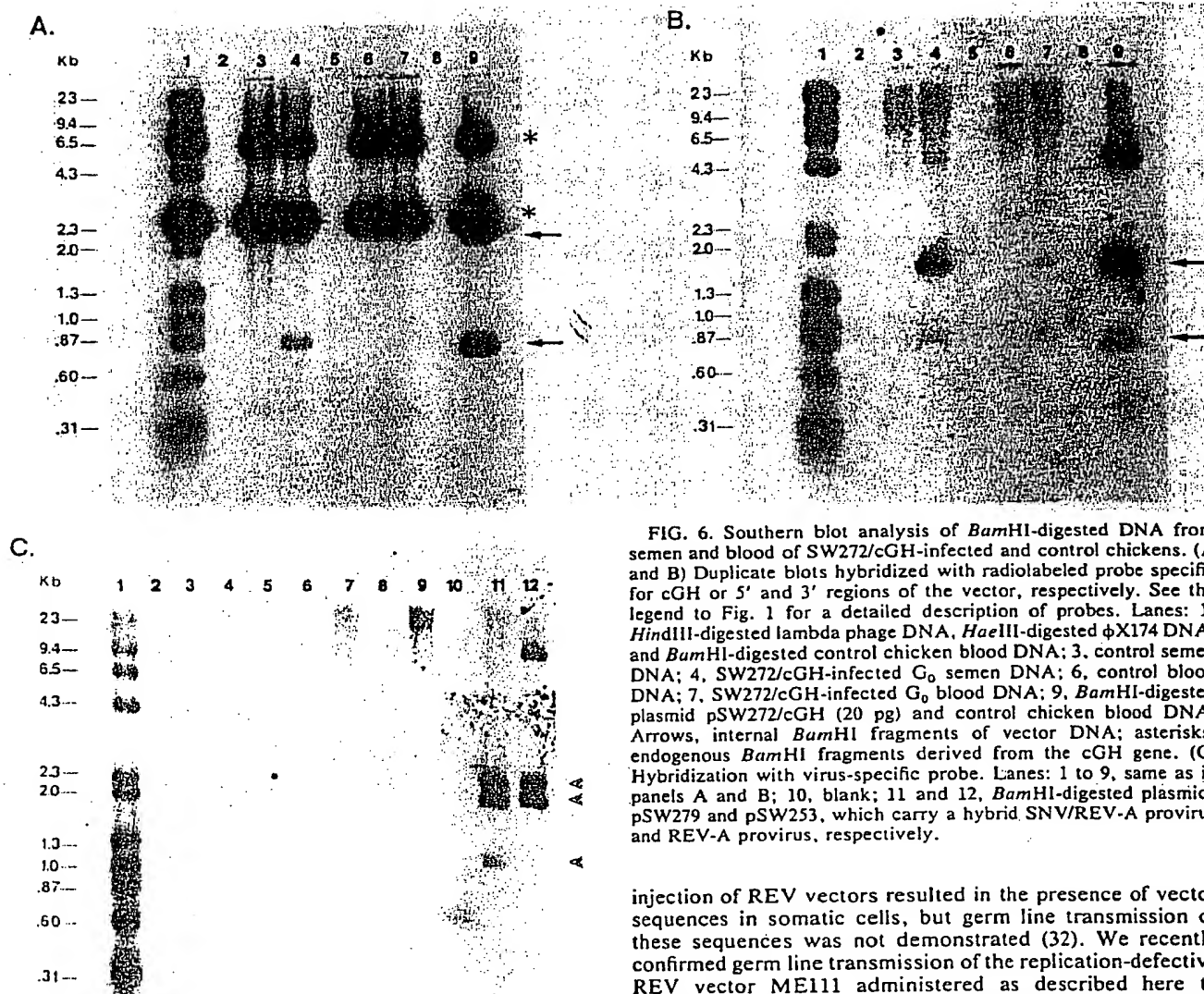


FIG. 6. Southern blot analysis of *Bam*HI-digested DNA from semen and blood of SW272/cGH-infected and control chickens. (A and B) Duplicate blots hybridized with radiolabeled probe specific for cGH or 5' and 3' regions of the vector, respectively. See the legend to Fig. 1 for a detailed description of probes. Lanes: 1, *Hind*III-digested lambda phage DNA, *Hae*III-digested  $\phi$ X174 DNA, and *Bam*HI-digested control chicken blood DNA; 3, control semen DNA; 4, SW272/cGH-infected G<sub>0</sub> semen DNA; 6, control blood DNA; 7, SW272/cGH-infected G<sub>0</sub> blood DNA; 9, *Bam*HI-digested plasmid pSW272/cGH (20 pg) and control chicken blood DNA. Arrows, internal *Bam*HI fragments of vector DNA; asterisks, endogenous *Bam*HI fragments derived from the cGH gene. (C) Hybridization with virus-specific probe. Lanes: 1 to 9, same as in panels A and B; 10, blank; 11 and 12, *Bam*HI-digested plasmids pSW279 and pSW253, which carry a hybrid SNV/REV-A provirus and REV-A provirus, respectively.

vector DNA fragment (data not shown) also seen in Fig. 5A. Vector sequences present in semen are not caused by contaminating blood cells since blood contains lower levels of vector DNA per microgram of total DNA, as shown by Southern blotting. Furthermore, blood cells were not detected in vector-positive semen subjected to microscopic examination nor could vector sequences be detected in negative control semen containing 1% vector-positive blood from a different bird. We did not observe any consistent *Bam*HI fragments representing junctions between cellular and vector sequences, probably because of the polyclonal makeup and low overall percentage of cells carrying the vector. No 1.4-kb *Bam*HI DNA fragment characteristic of replicating SNV was observed (40) (see Fig. 1).

Previous work with replication-competent derivatives of Rous sarcoma virus showed that infection of unincubated chicken embryos resulted in germ line insertion of proviral DNA (28, 29). In contrast, the same approach using competent REV resulted in somatic infection but did not lead to germ line insertion of proviral DNA (29). Similarly, follicular

injection of REV vectors resulted in the presence of vector sequences in somatic cells, but germ line transmission of these sequences was not demonstrated (32). We recently confirmed germ line transmission of the replication-defective REV vector ME111 administered as described here to chicken embryos (1). Breeding studies are now in progress to determine whether semen from the chickens infected as embryos with defective REV vectors encoding cGH can accomplish germ line transmission of the vector DNA to progeny.

**Conclusion.** Replication-defective REV vectors can introduce new genetic information into the chicken by infecting somatic stem cells of the embryo. Susceptibility of these stem cells to infection by REV vectors provides another approach to the in vivo study of avian development (4) and vector-mediated gene expression. The possible applications of this technology are numerous.

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## Transgenic production of a variant of human tissue-type plasminogen activator in goat milk: generation of transgenic goats and analysis of expression.

Ebert KM, Selgrath JP, DiTullio P, Denman J, Smith TE, Memon MA, Schindler JE, Monastersky GM, Vitale JA, Gordon K.

Tufts University School of Veterinary Medicine, North Grafton, MA 01536-1895.

We report the first successful production of transgenic goats that express a heterologous protein in their milk. The production of a glycosylation variant of human tPA (LATPA--longer acting tissue plasminogen activator) from an expression vector containing the murine whey acid promoter (WAP) operatively linked to the cDNA of a modified version of human tPA was examined in transgenic dairy goats. Two transgenic goats were identified from 29 animals born. The first animal, a female, was mated and allowed to carry the pregnancy to term. Milk was obtained upon parturition and was shown to contain enzymatically active LATPA at a concentration of 3 micrograms/ml.

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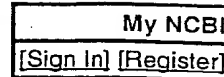
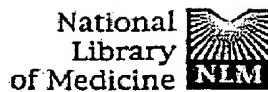
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1: Biotechnology (N Y). 1991 Sep;9(9):839-43.

Related Articles, Links

## Transgenic expression of a variant of human tissue-type plasminogen activator in goat milk: purification and characterization of the recombinant enzyme.

Denman J, Hayes M, O'Day C, Edmunds T, Bartlett C, Hirani S, Ebert KM, Gordon K, McPherson JM.

Genzyme Corporation, Framingham, MA 01701.

A glycosylation variant of human tissue-type plasminogen activator (tPA) designated longer-acting tissue-type plasminogen activator (LAtPA) was extensively purified from the milk of a transgenic goat by a combination of acid fractionation, hydrophobic interaction chromatography and immunoaffinity chromatography. This scheme provided greater than 8,000-fold purification of the protein, a cumulative yield of 25% and purity greater than 98% as judged by SDS gel electrophoresis. SDS gel electrophoresis revealed that the transgenic enzyme was predominantly the "two chain" form of the protease. The specific activity of the purified transgenic protein, based on the average of the values obtained for three different preparations, was 610,000 U/mg as judged by amidolytic activity assay. This was approximately 84% of the value observed for the recombinant enzyme produced in mouse C127 cells. Analysis of the transgenic protein indicated that it had a significantly different carbohydrate composition from the recombinant enzyme produced in C127 cells. Molecular size analysis of the oligosaccharides from the transgenic and C127 cell-derived LAtPA preparations confirmed their differences and showed that the mouse cell-derived preparation contained larger, complex-type N-linked oligosaccharide structures than the material produced in goat mammary tissue.

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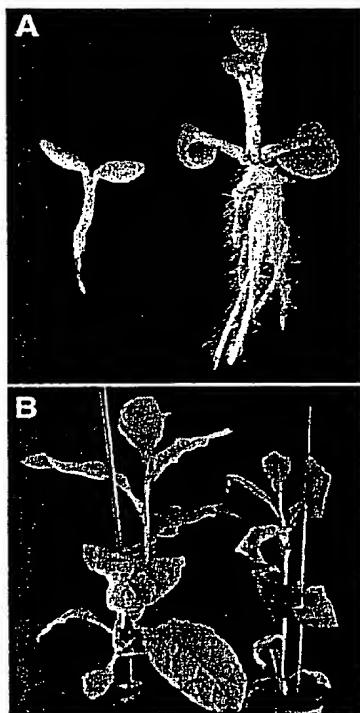
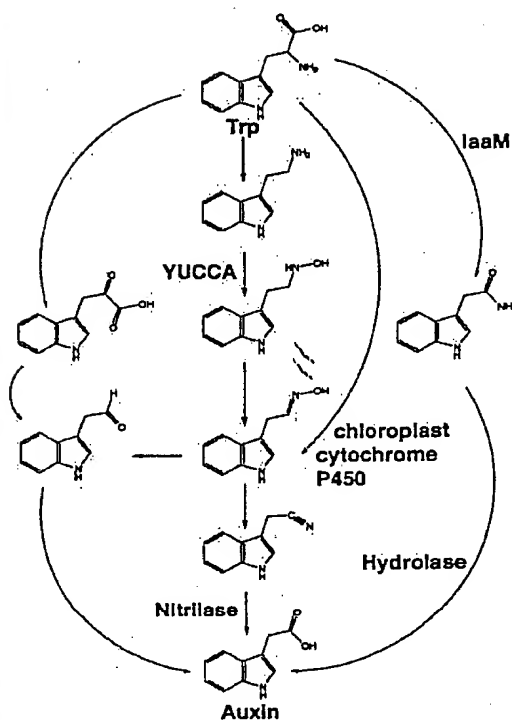


Fig. 4. (Left) YUCCA is involved in tryptophan-dependent auxin biosynthesis, and the YUCCA pathway is functional in other plants. (A) *yucca* is less sensitive to toxic tryptophan analogs. Wild-type (left) and *yucca* seedlings were grown on 0.5X MS medium containing 100-μM 5-mT for 10 days. (B) Comparison of wild-type (left) and transgenic tobacco plants overexpressing YUCCA. Fig. 5. (Right) YUCCA catalyzes a key step in auxin biosynthesis. Putative tryptophan-dependent auxin biosynthesis pathways and intermediates are shown (2). The indole-3-acetaldoxime intermediate was proposed recently (25).



may yield additional clues that can be used to elucidate the physiological roles of their mammalian counterparts.

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31. We thank M. Estelle for providing *iaaL* overexpression *Arabidopsis* lines, L. Barden for assistance with the art work, T. Dabi for help with tobacco transformation, and J. Perry and members of the Chory lab for useful comments. Supported by grants from NIH (2R01GM52413) and NSF (MCB9631390) to J.C., from NSF (MCB 9723823) to D.W., from NIH (R01GM36426) to J.R.C., from DOE (DE-FG02-00ER15079) and from Minnesota Agricultural Experiment Station and the Bailey Endowment for Environmental Horticulture to J.D.C., and from the Howard Hughes Medical Institute. Y.Z. is a HHMI Fellow of the Life Sciences Research Foundation; S.C. was partially supported by an NSF fellowship; C.F. was a fellow of the Human Frontier Science Program and the Swiss NSF. J.C. is an Associate Investigator of the HHMI.

12 October 2000; accepted 1 December 2000

## Transgenic Monkeys Produced by Retroviral Gene Transfer into Mature Oocytes

A. W. S. Chan, K. Y. Chong, C. Martinovich, C. Simerly, G. Schatten\*

Transgenic rhesus monkeys carrying the green fluorescent protein (GFP) gene were produced by injecting pseudotyped replication-defective retroviral vector into the perivitelline space of 224 mature rhesus oocytes, later fertilized by intracytoplasmic sperm injection. Of the three males born from 20 embryo transfers, one was transgenic when accessible tissues were assayed for transgene DNA and messenger RNA. All tissues that were studied from a fraternal set of twins, miscarried at 73 days, carried the transgene, as confirmed by Southern analyses, and the GFP transgene reporter was detected by both direct and indirect fluorescence imaging.

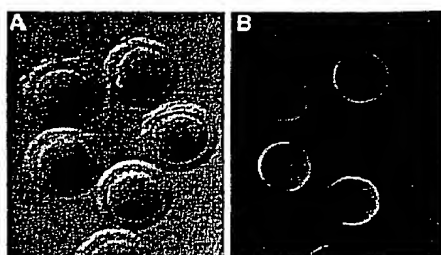
Although transgenic mice have been invaluable in accelerating the advancement of biomedical sciences (1–5), many differences between humans and rodents have limited their

usefulness (6–9). The major obstacle in producing transgenic nonhuman primates has been the low efficiency of conventional gene transfer protocols. By adapting a pseu-

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**Fig. 1.** Injection of VSV-G pseudotyped retroviral vector, enclosing the GFP gene and protein, into the perivitelline space of mature rhesus oocytes. (A) Transmitted light and (B) epifluorescence imaging of GFP carried within the vector particles. Magnification:  $\times 100$ .

dotyped vector system, efficient at up to 100% in cattle (10, 11), we circumvented problems in traditional gene transfer methodology to produce transgenic primates.

We injected 224 mature rhesus oocytes with high titer [ $10^8$  to  $10^9$  colony-forming units (cfu)/ml] moloney retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G (VSV-G pseudotype) into the perivitelline space (Fig. 1; Table 1; 10–12). The VSV-G pseudotype carried the GFP gene under the control of either the cytomegalovirus early promoter (CMV) [referred to as LNCEGFP-(VSV-G)] or the human elongation factor-1 alpha promoter (hEF-1 $\alpha$ ) [referred to as LNEFEGFP-(VSV-G)] (13). Because  $\sim 10$  to 100 pl was introduced into the perivitelline space, between 1 and 10 vector particles were introduced using LNCEGFP-(VSV-G) [ $10^9$  cfu/ml] and between 0.1 to 1 with LNEFEGFP-(VSV-G) ( $10^8$  cfu/ml). Oocytes were cultured for 6 hours before fertilization by intracytoplasmic sperm injection (ICSI). Vector particles incorporated into the oocyte in  $<4.5$  hours as imaged by electron microscopy (14). Fifty-seven percent ( $n = 126$ ) of embryos developed beyond the four-cell stage and 40 embryos were transferred to 20 surrogates, each carrying two embryos (Table 1). Rates for reproductive parameters are: fertilization [77% ICSI controls (15) versus 75% transgenesis], embryonic development [75% ICSI controls (15) versus 57% transgenesis], and implantation [66% ICSI controls (16) versus 25% transgenesis]. Most control ICSI pregnancies result in live offspring (83%) (16).

Five pregnancies resulted in the births of three healthy males (Table 1, Fig. 2). A set of



**Table 1.** Transgenesis efficiency in rhesus embryos, fetuses, and offspring.

Construct	VSV-G pseudotype		Overall
	LNCEGFP	LNEFEGFP	
Eggs injected with vector	157	67	224
Eggs then injected with sperm	157	65	222
Fertilization rate	108 (69%)	58 (89%)	166 (75%)
Embryonic development of fertilized eggs	85 (79%)	41 (71%)	126 (76%)
Embryos transferred (two/surrogate)	22	18	40
Number of surrogates	11	9	20
Pregnancies/surrogate	1* (9%)	4 (44%)	5 (25%)
Fetal losses	2 (100%)	1 (25%)	3 (50%)
Births	0	3	3
Transgenic	2 of 2	1 of 4	3 of 6
Transgenic birth/embryos transferred	0	1 (5.5%)	1 (2.5%)
Transgenic birth/pregnancies	0	1 (25%)	1 (20%)

\*Twin pregnancy.

fraternal twins miscarried at 73 days (150 to 155 days normal gestation) and a blighted pregnancy (implantation attempt without a fetus) also occurred. One fetal twin of the miscarriage was an anatomically normal male, while the other was largely resorbed in utero. The three births and the blighted pregnancy resulted from nine embryo transfers in which LNEFEGFP-(VSV-G) was used, whereas the twin pregnancy was established from 11 embryo transfers with LNCEGFP-(VSV-G) (Table 1).

Transgene integration, transcription, and expression from the newborns were examined in hair, blood, umbilical cords, placenta, cultured lymphocytes, buccal epithelial cells, and urogenital cells passed in urine, along with 13 tissues from the male stillborn, nine from the resorbed one, and specimens from the blighted pregnancy (17). Polymerase chain reaction (PCR) was performed with primer sets that covered the flanking region of the vector pLNC-EGFP or pLNEF-EGFP and the GFP

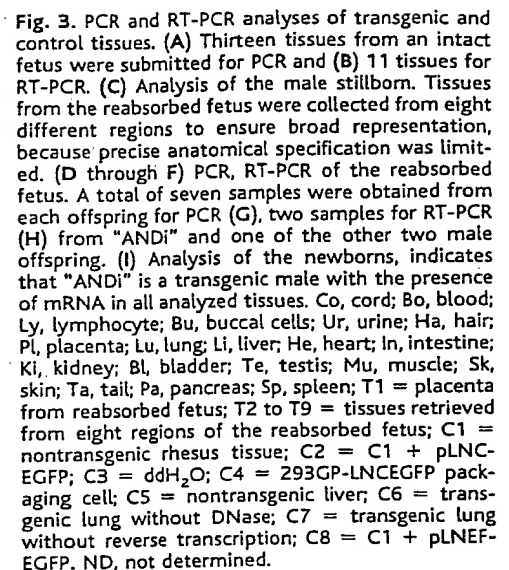
gene (18). One newborn, ANDi, showed the presence of the transgene in all analyzed tissues, and the transgene was present in all tissues analyzed from both stillbirths including placenta and testes (Fig. 3). Total RNA was extracted for standard reverse transcription followed by PCR amplification (RT-PCR) with primer sets specific for the transgene (18). Transgene transcription was demonstrated in all of the tissues in the fetuses and in the accessible tissues from the infant carrying the transgene (Fig. 3).

Southern blot analysis of 10 tissues from the male stillbirth and eight samples from the other twin demonstrated multiple integration sites into their genomic DNA (Fig. 4) (19). Vector integration was determined by PCR of placenta, cord, blood, hair, and buccal cells using a primer set specific for the unique retroviral long terminal repeat (LTR) regions indicative of successful provirus integration into the host genome (20, 21). This provirus sequence was found in one infant and both

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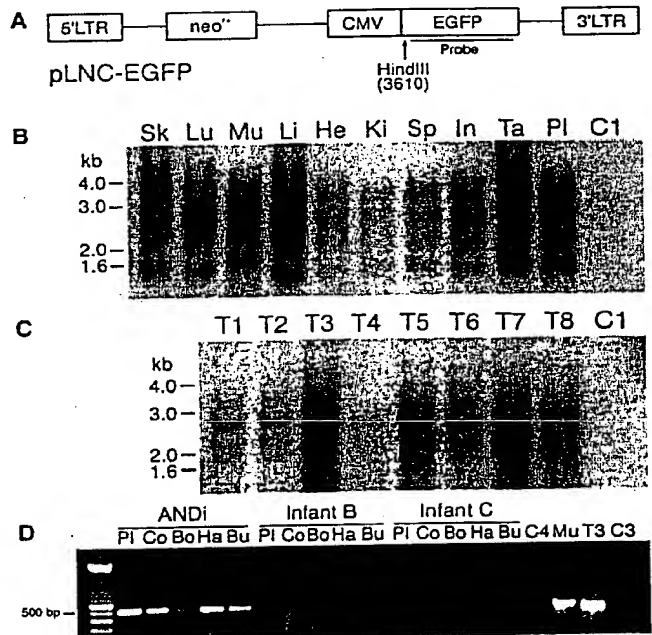


stillbirths (Fig. 4D). Infant welfare considerations limited tissue availability, and genomic DNA obtained was insufficient for Southern analysis. The male infant with the inserted transgene has been named "ANDI" (for "inserted DNA," in a reverse transcribed direction; Fig. 2A).

GFP direct fluorescence in the toenails and hair of the fetus, as well as the placenta (Fig. 2, B through F), provided further evidence of transgenesis. Colocalization between direct GFP fluorescence and indirect anti-GFP immunocytochemical imaging demonstrated that the GFP protein is found exclusively at the direct fluorescence sources (Fig. 2, D through F). Furthermore, neither direct fluorescein nor indirect rhodamine fluorescence was observed in controls (22). Because tissues from the fetus originated from the three germ layers, the timing of transgene integration may have occurred before implantation, perhaps even before the first DNA replication cycle (10). The high efficiency of this approach has been linked to the absence of the nuclear envelope in oocytes naturally arrested in second meiotic metaphase (10, 23).

The miscarriage is likely due to the twin pregnancy, which is rare and high-risk in rhesus. The twin stillbirth originated from the

Fig. 4. (A) Southern blot analysis of Hind III (single digestion site) digested genomic DNA. Full-length GFP labeled with [<sup>32</sup>P] was used as a probe to detect the transgene, which was detected in genomic DNA of the normal male stillborn (B) and reabsorbed fetus (C). Nontransgenic rhesus tissue was used as a negative control (C1) and pLNC-EGFP DNA as a positive control (not shown). Various sized fragments were demonstrated in tissues obtained from each. This result indicates multiple integration sites due to the use of a restriction enzyme with a single digestion site within the transgene. (D) Detection of the unique provirus sequence. A total of five tissues from each infant and two tissues from a male stillbirth and the reabsorbed fetus were submitted for PCR. Provirus sequence was they are transgenic. Abbr stillborn: T3, tissue from



fetus were submitted for PCR. Provirus sequence was detected in "ANDi" and the two stillbirths (42), which indicates that they are transgenic. Abbreviations are the same as those in Fig. 3. Mu, muscle from the male stillborn; T3, tissue from the reabsorbed fetus.

higher titer vector, whereas the three births, including the transgenic one, and the blighted pregnancy originated from the lower titer LNEFEGFP-(VSV-G) vector ( $10^8$  cfu/ml; Table 1). Although only one live offspring is shown to be transgenic, we cannot yet exclude the possibility of transgenic mosaics in the others. We have neither demonstrated germline transmission nor the presence of transgenic sperm; this must await ANDi's development through puberty in about 4 years. Vector titers and volume injected may play crucial roles in gene transfer efficiency. These offspring and their surrogates are now housed in dedicated facilities with ongoing, stringent monitoring.

Nonhuman primates are invaluable models for advancing gene therapy treatments for diseases such as Parkinson's (24) and diabetes (25), as well as ideal models for testing cell therapies (26) and vaccines, including those for HIV (27, 28). Although we have demonstrated transgene introduction in rhesus monkeys, significant hurdles remain for the successful homologous recombination essential for gene targeting (29). The molecular approaches for making clones [either by embryo splitting (30) or nuclear transfer (31–36)], utilizing stem cells (37–39), and now producing transgenic monkeys, could be combined to produce the ideal models to accelerate discoveries and to bridge the scientific gap between transgenic mice and humans.

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43. We thank J. C. Burns (University of California San Diego); B. True (University of Wisconsin-Madison); K. Wells (U.S. Department of Agriculture); Chiron Inc.; and all at the Oregon Regional Primate Research Center (ORPRC), especially M. Axthelm, J. Bassir, J. M. Cook, N. Duncan, M. Emme, J. Fanton, A. Hall, L. Hewitson, D. Jacob, E. Jacoby, A. Lewis, C. M. Luetjens, C. Machida, G. Macginnis, B. Mason, T. Swanson, D. Takahashi, K. Tice, J. Vidgoff, M. Webb, and S. Wong. Procedures approved by the Oregon Health Sciences University/ORPRC Animal Care and Biosafety Committees. Supported by NIH/National Center for Research Resources (NCRR) (ORPRC; M. S. Smith, Director) and grants (NCRR, National Institute of Child Health and Human Development to G.S.).

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## Categorical Representation of Visual Stimuli in the Primate Prefrontal Cortex

David J. Freedman,<sup>1,2,5</sup> Maximilian Riesenhuber,<sup>3,4,5</sup> Tomaso Poggio,<sup>3,4,5</sup> Earl K. Miller<sup>1,2,5\*</sup>

The ability to group stimuli into meaningful categories is a fundamental cognitive process. To explore its neural basis, we trained monkeys to categorize computer-generated stimuli as "cats" and "dogs." A morphing system was used to systematically vary stimulus shape and precisely define the category boundary. Neural activity in the lateral prefrontal cortex reflected the category of visual stimuli, even when a monkey was retrained with the stimuli assigned to new categories.

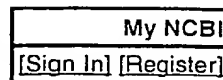
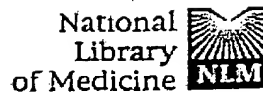
Categorization refers to the ability to react similarly to stimuli when they are physically distinct, and to react differently to stimuli that may be physically similar (1). For example, we recognize an apple and a banana to be in the same category (food) even though they are dissimilar in appearance, and we consider an apple and a billiard ball to be in different categories even though they are similar in shape and sometimes color. Categorization is fundamental; our raw perceptions would be useless without our classification of items as furniture or food. Although a great deal is known about the neural analysis of visual features, little is known about the neural basis of the categorical information that gives them meaning.

In advanced animals, most categories are learned. Monkeys can learn to categorize stimuli as animal or non-animal (2), food or non-food (3), tree or non-tree, fish or non-fish (4), and by ordinal number (5). The neural correlate of such perceptual categories might be found in brain areas that process visual form. The inferior temporal (IT) and prefrontal (PF) cortices are likely candidates; their neurons are sensitive to form (6–9) and they are important for a wide range of visual behaviors (10–12).

The hallmark of perceptual categorization is a sharp "boundary" (13). That is, stimuli from different categories that are similar in appearance (e.g., apple/billiard ball) are treated as different, whereas distinct stimuli within the same category (e.g., apple/banana) are treated alike. Presumably, there are neurons that also represent such sharp distinctions. This is difficult to assess with a small subset of a large, amorphous category (e.g., food, human, etc). Because the category boundary is unknown, it is unclear whether neural activity reflects category membership or physical similarity.

<sup>1</sup>Center for Learning and Memory, <sup>2</sup>RIKEN-MIT Neuroscience Research Center, <sup>3</sup>Center for Biological and Computational Learning, <sup>4</sup>McGovern Institute for Brain Research, <sup>5</sup>Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

\*To whom correspondence should be addressed. E-mail: [ekm@ai.mit.edu](mailto:ekm@ai.mit.edu)



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1: Biotechnology (N Y). 1991 Sep;9(9):844-7.

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## Generation of transgenic dairy cattle using 'in vitro' embryo production.

Krimpenfort P, Rademakers A, Eyestone W, van der Schans A, van den Broek S, Kooiman P, Kootwijk E, Platenburg G, Pieper F, Strijker R.

Department of Embryology, Gene Pharming Europe B.V., Leiden, The Netherlands.

We have combined gene transfer, by microinjection, with 'in vitro' embryo production technology, enabling us to carry out non-surgical transfer, to recipient cows, of microinjected embryos that have been cultured from immature oocytes. Using this approach, we have established 21 pregnancies from which 19 calves were born. Southern blot analysis proved that in two cases the microinjected DNA had been integrated in the host genome.

PMID: 1367358 [PubMed - indexed for MEDLINE]

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## Gene transfer into sheep



Simons, J. Paul; Wilmut, Ian; Clark, A. John; Archibald, Alan L.; Bishop, John O.; Lathe, Richard

Bio/Technology (1988), 6(2), 179-83 CODEN: BTCHDA; ISSN: 0733-222X. English.

Six transgenic sheep were formed by microinjection of ONA into the pronuclei of single-cell eggs. Three DNA constructs were microinjected: (1) pMK, which contains the mouse metallothionein-1 (MT) promoter linked to the herpes simplex thymidine kinase gene nTK) (2) BLG-FIX, which contains the  $\beta$ -lactoglobulin gene (BLG) linked to cDNA sequences encoding for human blood-coagulation factor IX and (3) BLG- $\alpha$ 1AT, which contains gene BLG linked to cDNA sequences encoding human  $\alpha$ 1-antitrypsin. The DNA in the transgenic sheep has not undergone rearrangement, as verified by hybridization assays. Hybridization intensities revealed the presence of single and multiple copies of constructs in the 6 lambs. Multiple copies had head-to-head and head-to-tail tandem arrangements. One of the offspring has the pMK construct, 4 of the offspring carry the BLG-FIX construct, and the last offspring carries the BLG- $\alpha$ 1AT construct. Offspring from these transgenic sheep also carry the transgenic genes.

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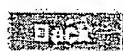


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## Expression of human anti-hemophilic factor IX in the milk of transgenic sheep

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Clark, A. J.; Bessos, H.; Bishop, J. O.; Brown, P.; Harris, S.; Lathe, R.; McClenaghan, M.; Prowse, C.; Simons, J. P.; et al.



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Bio/Technology (1989), 7(5), 487-92 CODEN: BTCHDA; ISSN: 0733-222X. English.

Transgenic livestock may prove useful for the large scale production of valuable proteins. By targeting expression to the mammary gland these proteins could be harvested from milk. To this end, a hybrid gene was designed to direct the synthesis of human anti-hemophilic factor IX to the mammary gland, and introduced into sheep. Two transgenic ewes, each carrying about 10 copies of the foreign gene, have been analyzed for expression. Both animals express human factor IX RNA in the mammary gland and secrete the corresponding protein into their milk.

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**X. RELATED PROCEEDINGS APPENDIX**

No Related Appeals and Interferences were cited in Section II, above.

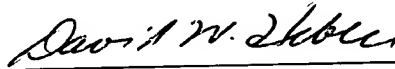
## **XI. CONCLUSION**

Appellants respectfully submit that, in light of the foregoing arguments, the Final Action's conclusion that claim 7 is not enabled is unwarranted. It is therefore requested that the Board overturn the Final Action's rejection of claim 7.

Respectfully submitted,

May 23, 2006

Date



David W. Hibler  
Agent For Appellants

Reg. No. 41,071

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